Discovery of Novel Anti-Inflammatory Proteins Inspired by Bone Marrow Mesenchymal Stem Cell Secretions

by

Jack Miles Milwid

S.B. Metallurgical and Materials Engineering,
Colorado School of Mines, 2005

S.M. Materials Science and Engineering,
Massachusetts Institute of Technology, 2007

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Signature of Author: _____
Jack Miles Milwid
Harvard-MIT Division of Health Sciences and Technology
April 28, 2011

Certified by: _____
Martin Yarmush, MD, PhD
Senior Lecturer of Surgery and Bioengineering, MGH
Thesis Supervisor

Accepted by: _____
Ram Sasisekharan, PhD
Director, Harvard-MIT Division of Health Sciences and Technology
Edward Hood Taplin Professor of Health Sciences & Technology and Biological Engineering

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I. Abstract

Bone marrow mesenchymal stem cells (MSCs) may soon become the first FDA-approved stem cell therapy for autoimmune and inflammatory disease. Our lab originally hypothesized that much of the therapeutic activity of MSCs may be attributed to molecules secreted by these cells. This thesis will test this hypothesis, with an emphasis on translational steps towards clinical product development, including the identification of novel proteins secreted by MSCs. The first part of the thesis consists of studies we performed to test whether MSC conditioned medium (MSC-CM) can treat rats undergoing cisplatin-induced acute kidney injury (AKI). When AKI rats were treated with MSC-CM, we observed a survival benefit and significant protection of renal function compared to controls. The second part of the thesis will describe the development of a device designed for sustained delivery of MSC secreted factors to dialysis-dependent AKI subjects. We tested these devices for cell function, stability and viability when subjected to conditions that model future clinical operation. Finally, inspired by the therapeutic capacity of MSC secreted factors, this thesis will conclude with the introduction of a new method that we developed to uncover novel anti-inflammatory proteins from MSCs. This method revealed four previously unidentified cytokine modulators, two of which we found significantly promote IL-10 and suppress TNF-α in mice challenged with endotoxin. When leveraged as novel therapeutics for lethal endotoxemic shock, these two most potent modulators protected mice and provided for a significant survival benefit compared to vehicle controls. Together, these results demonstrate the power of MSC secreted factors in the context of inflammatory disease, and propose new tactics for elucidating potent secreted products from cells.

Thesis supervisor: Martin Yarmush, MD, PhD
Title: Senior Lecturer of Surgery and Bioengineering, MGH

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V. Abbreviations

AKI: acute kidney injury
ALS: amyotrophic lateral sclerosis
ARF: acute renal failure
BMTx: bone marrow transplant
BUN: blood urea nitrogen
CD: cluster of differentiation
CFU-F: colony forming units-fibroblastic
CFU: colony forming unit
COPD: chronic obstructive pulmonary disease
CSF: cerebrospinal fluid
CTx: cell transplant
CVVH: continuous veno-venous hemodialysis
CyCAP: cyclophilin C-associated protein
DC: dendritic cell
DMSO: dimethyl sulfoxide
eGFP: enhanced green fluorescent protein
EKG: electrocardiogram
ELISA: enzyme-linked immunosorbent assay
EPS: enriched protein screening
FB: fibroblast
FBS: fetal bovine serum
FDA: United States Food and Drug Administration
FHF: fulminant hepatic failure
GALNT1: UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1
GCSF: granulocyte colony stimulating factor
GFR: glomerular filtration rate
GvHD: graft-versus-host disease
H&E: hematoxylin and eosin
HA: hydroxyapatite
HGF: hepatocyte growth factor
HSC: hematopoietic stem cell
MACS: magnetic activated cell sorting
MAGP2: microfibrillar-associated glycoprotein 2
MCP-2: monocyte chemotactic protein
MEM: minimal essential medium
MFAP5: microfibrillar-associated protein 5
MI: myocardial infarction
MLD: metachromatic leukodystrophy
MLR: mixed lymphocyte reaction
MoA: mechanism of action
MOA: method of administration
MOF: multiple organ failure
MRI: magnetic resonance imaging
mRNA: messenger ribonucleic acid
MS: mass spectrometry
MSC-CM: mesenchymal stem cell conditioned medium
MSC: mesenchymal stem cell
NK: natural killer
NOD: nonobese diabetic
NOD/SCID: non-obsese diabetic / severe combined immunodeficiency
OI: osteogenesis imperfecta
PBMC: peripheral blood mononuclear cell
PBPC: peripheral blood progenitor cell
PBS: phosphate-buffered saline
PCI: percutaneous coronary intervention
PCNA: proliferating cell nuclear antigen
PDGF-R: platelet derived growth factor receptor
PENK: proenkephalin
PES: polyethersulfone
PET: positron emission tomography
PK/PD: pharmacokinetics/pharmacodynamics
RAD: renal assist device

HTS: high throughput screening

ICAM: inter-cellular adhesion molecule

ICU: intensive care unit

IDDM: type I diabetes mellitus

IDO: indoleamine 2,3-dioxygenase

IHD: intermittent hemodialysis

IL: interleukin

iv: intravenous

LC: liquid chromatography

LGALS3BP: lectin, galactoside-binding, soluble, 3 binding protein

LIF: leukemia inhibitory factor

LPS: lipopolysaccharide

LVEF: left ventricular ejection fraction

mAb: monoclonal antibody

RAS: renin-angiotensin system

rhFGF: recombinant human fibroblast growth factor

ROS: reactive oxygen species

RST: renal support therapies

SCF: stem cell factor

TAF: Tumor-associated fibroblast

TCP: tricalcium phosphate

TCR: T cell receptor

TGF-β_1: transforming growth factor β_1

TNF-α: tumor necrosis factor α

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

Tx: transplantation

UCB: umbilical cord blood

VEGF: vascular endothelial growth factor

1. Introduction – Mesenchymal Stem Cells as a Source of Therapeutic Inspiration

In the past few decades, much has been learned about mesenchymal stem cells (a.k.a. bone marrow stromal cells, mesenchymal stromal cells and multipotent stromal cells, among other equivalents; MSCs). Clues as to their natural function, and a growing understanding of their therapeutic potential have come as a result of the tireless efforts of hundreds of research groups, thousands of clinicians and numerous companies across the globe. MSCs may soon become the first FDA-approved stem cell product for inflammatory and autoimmune diseases, and they have been shown to exhibit potent therapeutic activity in an impressive diversity of preclinical disease models.

While much has been accomplished within the field, as a therapeutic these cells are still in their infancy. Promising clinical data are encouraging, but there are still severe deficits in understanding even the most basic aspects of mechanisms of action (MoA). Pharmacokinetics / pharmacodynamics (PK/PD) analyses have thus far failed to clarify the fate of MSCs upon transplantation in human patients. And unlike single molecule therapeutics, MSCs behave dynamically upon transplantation, making thorough analysis as to the MoA extremely challenging. Future efforts will need to focus heavily on these analyses, as current methods for tracking and monitoring the fate of molecular therapeutics are insufficient for cell-based therapies. Without a thorough and tractable analytical scheme for optimizing MSC therapy, clinical trials are likely to sell them far short of what they might be capable of when properly administered. **Chapter 2** of this thesis will summarize the state-of-the-art in MSC therapy, and build the proper framework for understanding and contemplating this dilemma.

Surely, this dilemma has been a setback for the MSC transplantation community, and has led to the proposal of alternative modes of administration of MSC therapy. A few investigators have reported that MSC engraftment and differentiation are not necessary for

conveying therapeutic benefit, thereby eliminating the need for cell transplantation in certain contexts. We have since surmised that lengthy optimization efforts to maximize homing and engraftment might be unnecessary, and even suboptimal for conveying therapeutic benefit. Early work in our lab demonstrated that contrary to popular belief at the time, the molecules secreted by MSCs can recapitulate most, if not all of the therapeutic activity of transplanted cells in acute inflammatory organ failure syndromes (and likely in many other diseases of autoimmunity and inflammation as well). These first studies revealed the surprising finding that in a rat model of fulminant hepatic failure (FHF), whole cells administered intravenously were unable to provide any survival benefit, and yet when the same cell mass was lysed and the molecular contents relinquished, a survival trend was observed. Then, when the same cell mass was incubated with basal medium and the secreted factors collected and administered intravenously, a statistically significant survival benefit was conveyed. We subsequently developed an MSC-laden bioreactor that was connected to the bloodstream of FHF rats for continuous delivery of MSC secreted factors – this platform was shown to provide the best benefit of all. By pursuing this path, our lab discovered crucial information about the MoA of MSC transplantation and generated data to suggest that the secreted factors alone might be sufficient for therapy, thereby significantly simplifying the problem of PK/PD characterization, and suggesting an alternative modality for administration of MSC therapy, namely via MSC secreted factors. In addition, our lab was able to develop a novel platform for delivery of said factors that shows clinical promise. Subsequent work by other groups has validated our findings, and has demonstrated the importance of MSC paracrine signaling in a variety of preclinical disease models.

With this new understanding of the therapeutic potential of MSCs in hand, our lab has built upon this base and explored in much greater depth how MSC secreted factors could be leveraged to treat diseases. Soon after the initial results in FHF rats, Professor Martin Yarmush, Dr. Biju Parekkadan, Mr. Brian Miller and I founded a company to translate the bioreactor delivery platform for clinical use to treat patients with acute kidney injury (AKI) and other inflammatory organ failure syndromes. We have been working together ever

since to confirm with proof-of-concept studies that the secreted factors are suitable for AKI, and scale up of the rodent prototype device for use in humans is feasible. **Chapter 3** will describe the work we have done to enable successful scale up and translation of this system into a clinical product.

Having spearheaded primary efforts in translating this proprietary delivery system into a clinical product for AKI, we decided in parallel to begin further analysis of the secreted factors of MSCs with the hopes of uncovering some of the more important molecules involved in MSC therapy. Early studies in our lab indicated that MSCs secrete hundreds of different proteins, and likely numerous other biological molecules that could be contributing to the therapeutic activity of the heterogeneous mixture. Indeed, other groups have also demonstrated that there are many therapeutically relevant molecules secreted by MSCs. Each individually could have activity, and in combination these factors are likely responsible for the elegant and sophisticated physiological responses to MSC and MSC secreted factors therapy. We initially viewed this as a difficult, and potentially intractable problem – considering that MSCs secrete hundreds of proteins, arriving at an understanding of the critical combination that is necessary for therapeutic activity could require a career of dedicated research. In light of this challenge, we decided to instead look at the problem from the perspective of protein therapeutics discovery – here we have a cell that has demonstrated therapeutic activity in animals and humans, and recent evidence has suggested that the secreted molecules are responsible for most, if not all of the therapeutic activity. Hence, these secreted molecules, if mined correctly, could be a source of potentially therapeutic molecules. Inspired by the natural capabilities of MSCs to reverse many different disease with these molecules, we set out to attempt to mimic aspects of MSC therapy by isolating individual therapeutic factors that are made by MSCs, that when administered alone can recapitulate much of the activity of the heterogeneous mixture in MSC-CM in certain contexts.

Once we began to look at the problem in this light, we started to see a new problem that we would have to solve, one of methodology and utilization of state-of-the-art analytical

techniques for mining these secreted molecules for therapeutic candidates. Many have attempted to solve this problem in the past, using tried-and-true methods for scanning through the secreted contents of cells looking for therapeutic candidates. Generally speaking, the methods commonly used fall into the categories of chromatography and mass spectrometry – for separating and identifying molecular constituents, respectively. Naturally, we attempted to leverage these same techniques at first. However, after purifying the secreted factors from $1x10^9$ MSCs and failing to resolve single molecules, we decided to attempt a different approach.

The approach that we developed, enriched protein screening (EPS), is described in full detail in **Chapter 4**. In essence, we took a step back from analyzing the conditioned medium directly and instead asked whether the genes expressed by MSCs could be correlated with activity as determined by potency assessment. If so, and if we could refine our list of correlated genes using other cells and gene expression conditions that correspond to other levels of therapeutic activity, then perhaps we could generate a stratification scheme to enrich our gene list. From this enriched list, we could then screen recombinant proteins for activity in our potency assay, and then take those *in vitro* hits and subject them to further analysis in mice. The result of this analysis was the identification of four novel anti-inflammatory proteins expressed by MSCs, of which two were shown to exhibit potent anti-inflammatory capabilities that provided a significant survival benefit to mice challenged with the classical pro-inflammatory ligands, lipopolysaccharides. Using this methodology, we were able to uncover multiple novel anti-inflammatory proteins that show great promise as next-generation immunomodulatory drugs.

This thesis is intended to serve as an overview of the discoveries we have made over the past four years that highlight the great potential of MSC therapy, with a particular focus on the unique therapeutic capabilities of MSC secreted factors, and the development of a new methodology for discovering new protein drugs based on MSC secretions. We are hopeful that our proprietary delivery platform for the MSC secreted factors can make its way out of the laboratory and into the hands of clinicians to serve the many patients suffering from

inflammatory organ failure syndromes that are currently in desperate need of a new, transformative therapy. In addition, we are excited that our enriched screening method has thus far yielded several promising anti-inflammatory candidate therapeutics that might also have clinical applications if developed properly, and are enthusiastic to apply this method to other cell types to potentially reveal additional, potent therapeutic proteins.

2. Mesenchymal Stem Cells as Therapeutics

2.1. Abstract

Mesenchymal stem cells (MSCs) are multipotent cells that are being clinically explored as a new therapeutic for treating a variety of immune-mediated diseases. First heralded as a regenerative therapy for skeletal tissue repair, MSCs have recently been shown to modulate endogenous tissue and immune cells. Preclinical studies of the mechanism of action suggest that the therapeutic effects afforded by MSC transplantation are short-lived and related to dynamic, paracrine interactions between MSCs and host cells. Therefore, representations of MSCs as drug-loaded particles may allow for pharmacokinetic models to predict the therapeutic activity of MSC transplants as a function of drug delivery mode. By integrating principles of MSC biology, therapy, and engineering, the field is armed to usher in the next generation of stem cell therapeutics.

2.2. Introduction

The discovery of adult stem cells substantiated theories about the presence of regenerative populations of cells in developed organisms and has led to growing interest in the use of these cells as therapeutics. In particular, researchers are now exploring the use of mesenchymal stem cells (MSCs) in preclinical and clinical studies to resolve injury by enhancing endogenous repair programs, which represents a powerful new paradigm for treating human disease. From systemic administration of MSCs as an intravenous treatment to the delivery of their molecular secretions by extracorporeal devices, groups around the globe are focusing their attentions on this cell, seeking to harness its full therapeutic potential.

MSCs are an excellent candidate for cell therapy because (*a*) human MSCs are easily accessible; (*b*) the isolation of MSCs is straightforward and the cells can expand to clinical

scales in a relatively short period of time [1, 2]; (*c*) MSCs can be biopreserved with minimal loss of potency and stored for point-of-care delivery [3, 4]; and (*d*) human trials of MSCs thus far have shown no adverse reactions to allogeneic versus autologous MSC transplants, enabling creation of an inventory of third-party donor MSCs to widen the number of patients treated by a single isolation [5-7]. MSC transplantation is considered safe and has been widely tested in clinical trials of cardiovascular [8, 9], neurological [10, 11], and immunological disease [12, 13] with encouraging results. Unfortunately, within the past year, several of the pivotal lead trials either have undergone early termination or have failed to meet primary endpoints.

These results suggest an incomplete understanding of the underlying mechanism(s) of action of MSC therapy and point to the importance of further preclinical development. A more refined understanding of the natural functions of MSCs in the bone marrow may provide the basis for insight into their primary mode(s) of action. MSCs were first considered to be stromal progenitor cells in the bone marrow and were originally hypothesized to serve one primary role in their undifferentiated state: replenishment of stromal tissue in the bone marrow. However, MSCs and their stromal progeny also perform a number of alternative functions in the bone marrow, including the secretion of soluble mediators, which support hematopoiesis. These alternative functions are now being characterized in the context of MSC transplantation, whereby paracrine interactions between MSCs and host cells have been shown to relate directly to the therapeutic activity of MSCs.

Although it has not been definitively proven whether engraftment and differentiation of MSCs is necessary to convey this paracrine support, recent studies have suggested that less than 1% of systemically administered MSCs persist for longer than a week following injection [14, 15], and the observed benefits of MSC therapy may result from the relinquishment of their molecular contents upon administration. If this is true, engineering approaches may improve the optimization of MSC dosing and provide for alternative uses of MSCs to deliver drugs as active, dynamic delivery vehicles.

The aim of this review is to present an overview of the field of MSC therapy, with a particular focus on the various hypotheses concerning the mechanism(s) of action of MSCs. The majority of works cited focus on systemic administration of bone marrow–derived MSCs because this therapeutic modality has been explored more extensively than other means of administering MSC-based therapy. For alternative sources and modes of administration of MSCs, we refer the reader to other reviews [16-18]. We begin with a brief history of MSCs, from their initial discovery to current clinical programs using cell transplantation. We then revisit the biological origins and natural functions of MSCs and present the initial observations that laid the groundwork for therapeutic testing. Next, we describe retrospective studies that support the theory that MSCs can be conceptualized as drug-releasing particles that deliver their payloads in the course of hours to days. Finally, we propose the development of new pharmacokinetic analysis techniques based on this molecular-particle theory to motivate new clinical trial designs for therapeutic delivery of MSCs. Ultimately, such engineering analyses may better predict the biological activity of MSCs and leverage the therapeutic potential of these cells in a more rational way.

2.3. Origins and Developmental Biology of Mesenchymal Stem Cells

In this section, we begin with historical studies that first suggested the existence of MSCs (see **Figure 2.1**). We then focus on clinical studies that have used MSCs as an intravenous therapeutic. Finally, we describe the current methods used to characterize MSC populations and highlight the known differentiated functions of MSCs in their native, in vivo microenvironments.

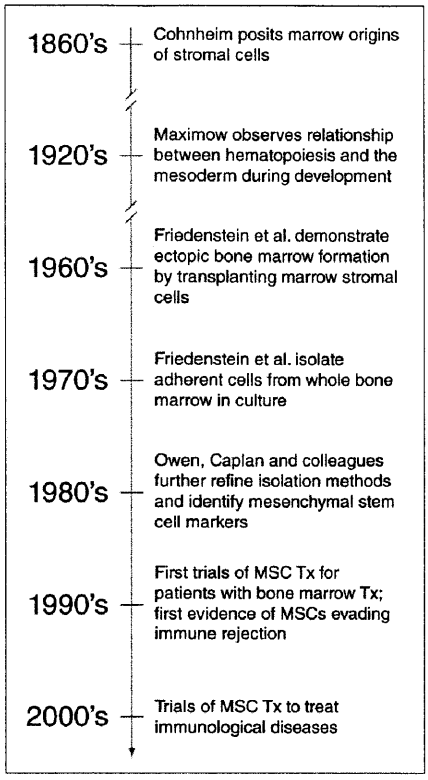


Figure 2.1 – A brief history of mesenchymal stem cells. Abbreviation: Tx, transplantation.

2.3.1. History of Mesenchymal Stem Cells: From Discovery to Clinical Therapy

The existence of a stromal precursor giving rise to mesodermal cells in the bone marrow was originally theorized in the nineteenth century [19]. Cohnheim hypothesized a bone marrow origin of fibroblasts implicated in distal wound healing [20]. In the early twentieth century, Maximow described the essential relationship between newly forming blood components and the mesoderm during embryogenesis. He initially postulated the importance of the marrow stromal tissue in supporting the development and maintenance of blood and hematopoietic organs [21]. These observations made by Cohnheim and Maximow offered the first indication of a reservoir of stromal cells in the bone marrow that were involved in the natural healing response and hematopoiesis.

In vivo transplantation of bone marrow elements in the 1960s demonstrated that stromal precursors are directly involved in the formation of skeletal tissue cells. Friedenstein et al.

first demonstrated that stromal cells could be isolated from whole bone marrow aspirates based on differential adhesion to tissue culture plastic—a method still widely practiced to isolate MSCs. These stromal cells were originally described as adherent, clonogenic, nonphagocytic, and fibroblastic in nature, with the ability to give rise to colony forming units-fibroblastic (CFU-F) [22]. Transplantation of these marrow stromal cells under the kidney capsule or in subcutaneous space led remarkably to the formation of ectopic marrow. Decomposition of the origins of ectopic marrow cells revealed donor-derived bony trabeculae, myelosupportive stroma, and adipocytes and host-derived hematopoietic cells that colonized and matured within the space [23-25]. Experiments performed with dermal fibroblasts or differentiated connective tissue cells failed to recapitulate the same histological image, demonstrating that this was a phenomenon specific to the marrow stroma. These seminal experiments illustrated an "organizing" function of MSCs similar to other lymphoid stromal cells and suggested that MSCs were a precursor to bone marrow connective-tissue cells.

In the late 1980s, Maureen Owen and Arnold Caplan elaborated on Friedenstein's initial work and proposed the existence of an adult stem cell that was responsible for mesengenesis [25, 26]. Owen et al. further characterized the marrow stroma and illustrated the heterogeneity of the population [25, 27-30]. At the same time, Caplan and colleagues hypothesized that a subpopulation of the marrow stroma was developmentally linked to the mesenchymal tissues he had been studying during chick embryogenesis [26, 31]. In addition, they identified the first set of MSC-expressed antigens that react with SH2 (CD105) and SH3 (CD73) monoclonal antibodies. Caplan and colleagues coined the term mesenchymal stem cell to describe this subtype of marrow stromal cells involved in the process of mesengenesis [7, 32, 33].

Shortly following the discovery of methods for isolating and culturing MSCs, the field began to grow rapidly, and many groups began to explore their therapeutic uses. Only a few years after MSCs were identified, human trials were commenced to evaluate safety and efficacy of MSC therapy. Initially, autologous MSCs were explored to aid in the engraftment and

recovery of hematopoiesis after ablation and bone marrow transplantation for the treatment of cancer [34, 35]. Concurrently, researchers conducted a number of groundbreaking studies that leveraged the therapeutic potential of allogeneic MSC transplants to treat children with osteogenesis imperfecta, a genetic disorder of skeletal dysplasia [36, 37]. Shortly thereafter, more studies were performed to investigate the utility of allogeneic MSCs to treat patients with Hurler syndrome and metachromatic leukodystrophy [38]. The focus of these early studies was predicated upon the fact that MSCs functioned as stromal stem cells and therefore might be best suited to treat diseases and conditions afflicting connective and hematopoietic tissue. These first studies were important because they provided preliminary evidence of the safety of MSC therapy as well as the basis for good manufacturing processes to generate MSCs on a clinical scale.

More recently, groups around the world have investigated MSC transplantation for the treatment of myriad diseases based on a newfound appreciation for MSCs' pleiotropic functions that enhance endogenous repair and attenuate immunological dysfunction. Examples of clinical trial designs are provided in **Table 2.1.** Currently, there are 79 registered clinical trial sites for evaluating MSC therapy throughout the world (http://clinicaltrials.gov/). The United States has the highest concentration of registered trial sites at 28, but the rest of the world accounts for more than half of the total number (19 in Europe, 16 in China, 5 in the Middle East, 4 in India, 3 in Canada, 2 each in Africa and Japan, and 1 in Australia), indicating strong international interest in MSCs as a potential therapy. The majority of trials are sponsored by academic medical centers exploring novel applications of MSCs to treat conditions as diverse as critical-limb ischemia (NCT00883870), spinal cord injury (NCT00816803), and liver cirrhosis (NCT00420134).

Table 2.1 – Examples of clinical trials of mesenchymal stem cell therapy reported in the academic literature

Indication	Enrollment (N_{total}; $N_{control}$, N_{cells})	Trial Design	Cell Mass MOA	Measured Parameters and Follow-up Time after CTx	Study Conclusions	Ref.
BMTx	(23; 15, 8)	Local advanced or metastatic breast cancer with high-dose chemotherapy and PBPC Tx given Ctx at 1 or 24 hours later	1.5-3.9x10^6 CD34+ cells/kg and 2.2x10^6 MSCs/kg	Neutrophil and platelet counts (daily), CFU (42 days), Mortality (100 days)	(1) Prompt hematopoietic engraftment of 8 days (2) 70% CFU recovery (3) One patient death after 100 days	[35]
OI	(7; 2, 5)	Diagnosed severe type III OI with similar growth rates	5.7-7.5 x10^8 bone marrow cells/kg i.v.	Growth analysis (6 months)	Increased growth rates that slowed over time	[36]
MLD	(5; 1, 4)	Patients with successful matched bone marrow transplants	2-10 x10^6 MSCs/kg iv	Nerve conduction velocity Mental/Physical Exams	(1) No toxicity (2) Improved nerve conduction velocity (3) No clinical change in overall mental or physical status	[38]
ALS	(7; 5, 2)	Diagnosed ALS with severe lower limb and mild upper limb impairment	MSCs suspended in CSF infused into T7-T9 exposed spinal cord	MRI (3, 6 months), Neuromuscular exam (3 months)	(1) No adverse reactions (2) No structural MRI changes (3) Mild increase in muscle strength of a lower limb muscle group	[39]
MI	(69; 35, 34)	CTx 10 days after PCI	48-60 x10^9 MSCs into coronary artery lesion	Echocardiography (monthly) PET (3, 6 months) EKG (3 months)	(1) Decreased akinetic, dyskinetic, hypokinetic segments (2) Increased wall movement at infract (3) LVEF higher	[9]
GvHD	(56; 10, 46)	Open-label, multicenter trial of hematological cancers patients treated with CTx 4 days prior to haploidentical BMTx	1, 5, or 10 x10^8 MSCs/kg iv	Hematopoietic Recovery (daily) Acute GVHD	(1) Prompt hematopoietic engraftment in most patients (2) 23/46 patients did not undergo acute GVHD with 11 showing longer relapse time	[12]

Abbreviations: MOA, method of administration; CTx, cell transplant; MI, myocardial infarction; PCI, percutaneous coronary intervention; MSC, mesenchymal stem cell; PET, positron emission tomography; EKG, electrocardiogram; LVEF, left ventricular ejection fraction; GvHD, graft-versus-host disease; BMTx, bone marrow transplant; MLD, metachromatic leukodystrophy; OI, osteogenesis imperfecta; ALS,

amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; PBPC, peripheral blood progenitor cell; CFU, colony forming unit; Tx, transplantation; iv, intravenously.

Osiris Therapeutics, Inc., founded in 1991, has played a pivotal role in the past decade in shaping the direction of research and development of MSC-based therapies. Concurrent with emerging theories regarding the immunological activity of MSCs, which we discuss in greater depth below, Osiris has developed a clinical trial program to explore the therapeutic utility of MSCs in humans. It has pioneered studies to investigate systemic administration of MSCs as a therapy for steroid-refractive graft-versus-host disease (GvHD), Crohn's disease, type I diabetes mellitus (IDDM), myocardial infarction (MI), and chronic obstructive pulmonary disease (COPD). Because early trials established a good record of safety for direct MSC injection, many of these studies are currently in Phase II or Phase III trials, and MSCs are already granted expanded access for use in pediatric steroid-refractive GvHD by the United States Food and Drug Administration (FDA). However, in the past year, Osiris has reported that several of its Phase II and Phase III studies were either prematurely terminated or failed to meet primary endpoints (http://www.osiristx.com). In March 2009, initial results for the Crohn's disease trial found a greater-than-expected placebo response, which led the company to cease recruitment after enrolling 210 patients in a Phase III study. In June 2009, six-month interim data of the Phase II COPD trial were announced: They indicated a statistically significant decrease in systemic inflammation as measured by C-reactive protein in MSC-treated patients but no statistical improvement of lung function. In September 2009, Osiris reported the results of two Phase III double-blind, placebo-controlled trials for adult GvHD in which MSC transplants were tested either as a first-line therapy or in patients that were refractory to standard medical treatment. Both trials showed an insignificant improvement in mortality compared with placebo at a 28-day endpoint (first-line therapy: N=192; 45% response rate with MSCs versus 46% response rate with placebo; refractory therapy: N = 260; 35% with MSCs versus 30% with placebo). Stratification of the cohorts based on subclasses of GvHD have yet to be reported but may show benefit in specific patient populations. Nevertheless, the results of these trials may curtail efforts to develop and validate MSC therapy in humans.

These results may indicate an incomplete understanding of the mechanisms of action of MSCs, and therefore an inefficient administration of the cells that does not best convey therapeutic benefit. Many clinical trials entail the administration of MSCs systemically and assume that MSCs engraft and provide long-term support by either directly replenishing damaged tissue or interacting with neighboring cells to promote endogenous repair. Currently, it is widely debated whether MSC engraftment, proliferation, and/or differentiation are necessary for therapeutic benefit. Many new studies are now implicating paracrine signaling as the primary mechanism of action, and a few studies have even demonstrated that direct injection of the molecules secreted by MSCs can provide an improved benefit above and beyond what is conveyed by transplanted whole cells [40-42]. In later sections, we discuss this issue further and present a new theory as to how MSCs may be conveying therapeutic benefit without engraftment and differentiation—i.e., how they instead may be acting as dynamic drug delivery vehicles.

2.3.2. Phenotype and Multipotency Analysis for the Identification of Mesenchymal Stem Cells

One of the most elusive problems in MSC biology has been the identification of a single marker that distinguishes a purified population of MSCs with a uniquely defined set of functional properties. Without such an identifier, definitively comparing putative MSCs from different tissues has been challenging. Exhaustive phenotypic analysis has therefore been necessary to distinguish MSCs from other cells that exhibit similar fibroblastic, adherent characteristics in culture. **Table 2.2** summarizes the current phenotypic tests used to identify MSCs.

Table 2.2 – Phenotype tests for mesenchymal stem cells and known MSC phenotypic characteristics

Phenotype Test	Known MSC Phenotype	Methods Used	Ref.
Colony Formation	Will form fibroblastic colonies after isolation	CFU-F assay	[22, 43]
Immunophenotype	CD11-, CD14-, CD18-, CD31-, CD34-, CD40-, CD45-, CD56-, CD80-, CD86-, MHCII- CD29+, CD44+, CD71+, CD73+, CD90+, CD105+, CD106+, CD120a+, CD124, CD166+, Stro-1+, ICAM-1+, MHCI+	FACS	[44, 45]
In Vitro Multipotency	Will differentiate down multiple pathways: 1. Osteogenic 2. Chondrogenic 3. Adipogenic	Induction via specialized media	[46]
Ectopic Marrow Formation	Will form ectopic bone marrow in the presence of bone minerals	Subcutaneous transplantation, kidney capsule transplantation, diffusion chamber transplantation	[47, 48]
In Vivo Multipotency	Subset of MSCs, termed MAPCs, are capable of contributing to all somatic cell types in mice	Blastocyst transplantation	[49]

[a]Abbreviations: CFU-F, colony forming units-fibroblastic; FACS, fluorescent activated cell sorting; MAPC, multipotent adult progenitor cell.

As described in **Table 2.2**, the phenotype of MSCs is defined in part by the multipotency of these cells in culture and in vivo. Determining in vivo multipotency is a powerful tool for assessing MSC phenotype. As far back as Friedenstein, ectopic transplantation of MSCs has been used to determine whether the MSC-like cells are capable of inducing bone and marrow formation [48, 50, 51]. Perhaps the most rigorous method for determining the "stemness" of an MSC population involves the injection of MSCs into the blastocyst of a mouse and studying the developmental progeny from the injected cell populations [49]. This method is not commonly used, however; although it was shown to be useful for a subset of MSCs, it does not necessarily reflect the phenotype of MSCs in general.

2.3.3. Embryonic and Adult Sources of Mesenchymal Stem Cells

The developmental precursor of MSCs has been difficult to identify because MSCs have no distinguishing features to track in vivo. A number of studies support the concept that the typical sites of developmental hematopoiesis, including the placenta, aorta-gonad-mesonephros, and fetal liver, are also populated by embryonic MSCs [52-54]. These cells are originally independent of interactions with hematopoietic stem cells and can be found in the embryonic circulation at early stages of ontogeny [55]. Counterintuitively, a novel embryological source of MSCs has been identified in the cranial neural crest. Through in situ methods with fluorescent reporting proteins, one group demonstrated a transient proliferation of Sox-1+ cells originally from the neuroepithelium that display multipotency and that transitioned through a neural crest stage to give rise to adult MSCs [56]. Cells with multilineage differentiation potential and cytoskeletal elements reminiscent of adult MSCs can be isolated from the first branchial-arch, ectomesenchymal cells that give rise to the orofacial connective tissue [57-59]. These results are consistent with the promiscuous expression of neural proteins in MSCs in their basal state [60, 61]. To date, no studies have determined the genetic events that guide the lineage specification of embryonic precursors into MSCs. Such genomic profiling can ultimately lead to new ways to derive these cells from embryonic stem cells or other ontogeny-related cell types.

Although the bone marrow has been established as the primary source of MSCs, because of the invasive nature of bone marrow aspiration, efforts are underway to identify other abundant and reliable sources of MSCs for clinical purposes. The isolation of MSCs from peripheral sources such as umbilical cord blood (UCB) [62, 63], placental tissue [52-54], and adipose tissue [46] has been reported with cells displaying similar immunophenotypes and multipotency, although other contradictory studies report the absence of MSCs in these peripheral locations [64, 65]. Whether there is a definitive relationship between these cells from various sources is unclear because rigorous studies of in vivo multipotency have yet to be done. Furthermore, it is important to be wary of interpretations of CFU-F analysis of MSCs from sites other than the bone marrow, given that many adherent and clonogenic

fibroblastoid cells exist in nonhematopoietic tissues. Although phenotypically similar, or even identical in some cases, MSCs derived from different tissues have been shown to exhibit variable function and activity [66]. It is thought that the MSC niche, unique to each tissue of origin, is at the root of these variations.

2.3.4. Mesenchymal Stem Cell Localization and Mobilization In Vivo

The physical location, or niche, of a stem cell provides invaluable information about its role and interactions within the tissue (see **Figure 2.2**). Bone marrow MSCs have been explored for therapeutic use more extensively than any other subtype, and the native functions of these cells in the bone marrow have been studied in hopes of revealing clues about their therapeutic activity [67, 68]. The MSC niche has been difficult to locate and even harder to observe dynamically because no unique MSC marker has been identified and because the marrow cavity is difficult to probe in vivo. That said, based on correlations between immunophenotype and ex vivo CFU-F assays, evidence supports the idea that MSCs exist in perivascular locations [69, 70]. This theory is consistent with the observations that (*a*) MSCs are presumably found in many tissue types including synovium, periosteum, adipose, UCB, and placenta [71, 72]; (*b*) the number of MSCs in a given tissue scales with the density of microvasculature; (*c*) MSCs secrete factors that promote vasculogenesis and endothelial stabilization [73]; and (*d*) they may exhibit different functional characteristics depending on the derivative tissue type [66]. Their stromal counterparts may differentiate and migrate from this space to reside on the albuminal side of marrow sinusoids and form a three-dimensional network that invests the capillary bed.

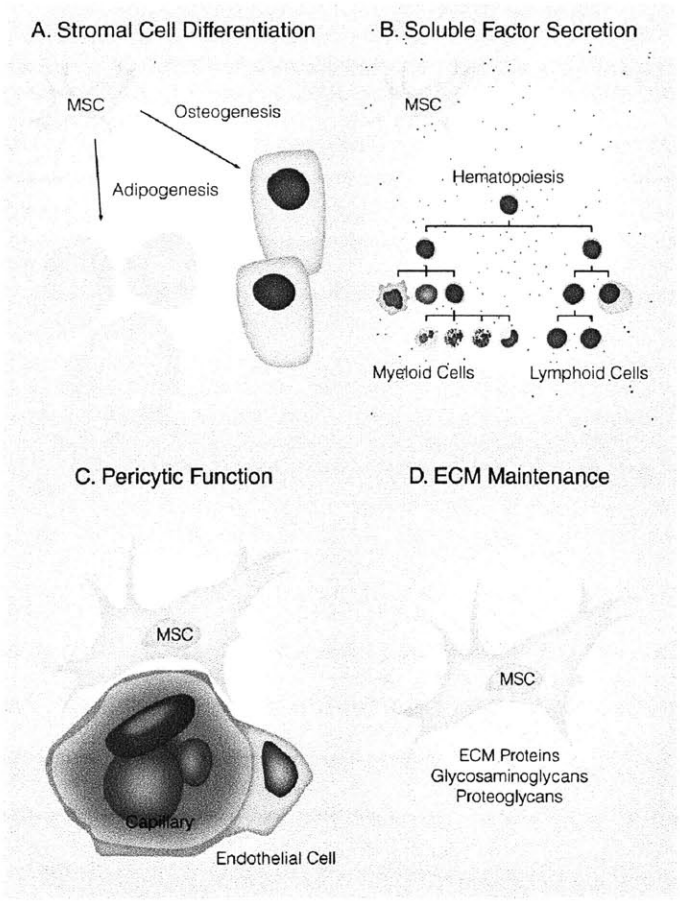


Figure 2.2 – Natural functions of mesenchymal stem cells in the bone marrow. (*a*) MSCs can differentiate into skeletal tissue cells within the marrow cavity. (*b*) MSCs secrete a number of soluble factors that are involved in hematopoietic development. (*c*) Given their purported perivascular localization, MSCs may serve cellular functions similar to pericytes that surround bone marrow sinusoids. (*d*) MSCs maintain the mechanical microenvironment of the marrow by secreting and remodeling ECM. Abbreviations: MSC, mesenchymal stem cell; ECM, extracellular matrix.

Adventitial reticular cells, or pericytes, that have fibroblastic extensions projecting into the lumen of sinusoids, are likely the in vivo surrogate of CFU-F, although single-cell analytical studies have not been performed [68, 74, 75]. These pericytes share a similar surface and intracellular protein expression pattern with MSCs, which implies that the cells are ontologically related [76]. This location has been reproduced in artificial systems as well. Ectopic stromal cells displaying PDGF-R, NG2, and high expression of CD146 are typically localized in perisinusoidal regions [77]. In addition, tissue-engineered constructs juxtaposing MSCs and endothelial cells form long-lasting vascular structures, with MSCs naturally displaying pericytic phenotype and function [78]. Such localization suggests that MSCs may be intimately involved in angiogenesis, wound healing, and interactions with blood-borne entities.

Given their purported perivascular location, the question of whether MSCs mobilize into the bloodstream during health and disease is an important one that has not been answered. It is unlikely that *bona fide* MSCs circulate peripherally because of their limited numbers (~0.01% of mononuclear bone marrow cells); it is more likely that they produce lineage-restricted cell types that home to tissues as a mechanism of nonparenchymal cell replenishment during injury. Fibrocytes are circulating bone marrow–derived cells (~0.1–0.5% of nonerythrocytic cells in peripheral blood) that phenotypically resemble a hybrid of monocytes and fibroblasts expressing type I collagen and the surface markers CD11b, CD13, CD34, and CD45RO [79]. In sex-mismatched bone marrow chimeras, these cells were found to be the progeny of a radioresistant precursor from the bone marrow [80]. During injury, fibrocytes were rapidly and specifically found in the areas of inflammation [80], fibrosis [81], and cancer [82-85], where they are thought to mature into tissue-resident myofibroblasts [86]. They express chemotactic receptors such as CCR3, CCR5, CCR7, and CXCR4 and are absent of CCR4, CCR6, and CXCR3 [79]. Interestingly, fibrocytes express surface molecules such as major histocompatibility complex class II, CD80, and CD86 and were shown to present pulsed antigens to naïve T cells in an efficient manner when compared with monocytes and dendritic cells, although this was not verified in vivo [87]. These cells have functional and phenotypic resemblance to bone marrow resident MSCs; therefore, refining current theories to distinguish the MSC-fibrocyte axis may lead to a greater understanding of each cell type during health and disease.

2.3.5. The Interaction between the Bone Marrow Stroma and Hematopoiesis

MSCs exist within the bone marrow as a precursor to connective-tissue components that act primarily as supportive elements to hematopoiesis. The essential functions of MSCs and their precursors within the marrow can provide insight into mechanisms involved when these cells are used in a therapeutic context. The initial appreciation for the important interaction between stromal cells and hematopoietic cells was obtained from the analysis of two different spontaneous mutations in mouse colonies that led to the same anemic

phenotype. Analysis of these mutant mice revealed that a stromal cell ligand known as stem cell factor (SCF) and its associated receptor, c-kit, found on hematopoietic stem cells (HSCs) was essential for the maintenance of HSCs. Other cell-cell interactions between MSC progeny, such as osteoblasts and HSCs, have also proved essential for HSC self-renewal [88, 89]. Moreover, stromal elements secrete a number of insoluble and soluble species within the marrow space that promote the growth and differentiation of hematopoietic cell lineages [90-93].

Experimentally, MSCs can act as a surrogate feeder layer and promote the self-renewal and differentiation of HSCs in long-term colony-initiating culture and CFU assays [94]. Two types of culture techniques utilizing stromal cell layers and defined chemical supplements allow for the establishment of lymphoid and myeloid cells in vitro. The Whitlock-Witte method cultures bone marrow cells on a confluent layer of irradiated stromal cells with a low-serum-containing medium without corticosteroids [95, 96]. It is a lymphoid culture system, which supports the growth of B lymphocytes and which, with some modifications in culture parameters, can also allow selective proliferation and differentiation of all developmental stages of pre-B cells and B lymphocytes. A myelopoietic culture system, known as Dexter cultures, maintains myeloid progenitor cells and differs from Whitlock-Witte cultures by using high concentrations of serum and hydrocortisone and lower incubation temperatures [97, 98]. Collectively, the marrow stroma can direct the differentiation of lymphoid and myeloid cells in vitro, and it is likely that the mechanisms underlying this directed differentiation will be relevant to the immune response to MSCs in vivo.

2.4. Preclinical and Clinical Applications of Mesenchymal Stem Cell Therapy

An extra level of biological understanding is often gained during testing of a new therapeutic. The evolution of MSC therapy over the years reflects a transformation in how

investigators view these cells and their best-suited clinical applications. Initially heralded as stem cells, MSCs were first evaluated for regenerative applications. MSCs have since been shown to directly influence the innate and adaptive arms of the immune system [99], enhance proliferation of epithelial cells [100], and promote neovascularization of ischemic tissues [73]. These observations have prompted a new age of MSC transplantation as a treatment for immune-mediated and tissue-sparing diseases. In this section, we capture this paradigm shift and focus on important studies that contextualize MSCs as a therapeutic for regenerative medicine and inflammatory diseases. Furthermore, we discuss the potential side effects that should be considered when MSCs are used.

2.4.1. Mesenchymal Stem Cells for Regenerative Medicine and Inflammatory Diseases

MSCs are currently being explored for use in humans because of their potent ability to treat many devastating diseases in animals (see **Table 2.3**). Although the primary mechanisms of action have not been fully elucidated, studies indicate that MSCs can act on several levels of endogenous repair to bring about resolution of disease. MSCs have been shown to protect cells from injury and directly promote tissue repair [101, 102]. When administered to treat animals undergoing acute kidney injury, MSCs prevent apoptosis and elicit proliferation of renal-tubule epithelial cells in a differentiation-independent manner [103-105]. When injected into the myocardium after infarction, MSCs can reduce the incidence of scar formation [106-108]. When administered to prevent the onset of IDDM, MSCs protect β-islets from autoimmune attack; when administered after onset of the disease, they promote temporary restoration of glucose regulation, suggesting protection and repair of damaged islet tissues [109].

Table 2.3 – Animal studies of diseases shown to respond to administration of mesenchymal stem cells

Disease	Animal Model(s)	Method of Administration	Evidence of MSC Efficacy	Ref.
Acute Kidney Injury	Rodent Cisplatin Ischemia / reperfusion	Intravenous infusion	Decreased serum creatinine Decreased apoptosis Increased epithelial proliferation Suppression of pro-inflammatory cytokine gene expression	[104, 105, 110-112]
Myocardial Infarction	Rodent LAD ligation Pig Temporary LAD occlusion	Intravenous infusion Intramyocardial transplantation	Reduction in scar formation Improvement in cardiac function Differentiation of MSCs into functioning myocardium	[106-108, 113, 114]
Type I Diabetes Mellitus	Rodent NOD mice Streptozotocin	Intravenous infusion	Partial restoration of glucose management Reduction in anti-insulin T cells Prevention of FOXP3+ cell apoptosis	[109, 115-118]
Graft vs. Host Disease	Rodent HLA-mismatched bone marrow transplantation	Intravenous infusion Intravenous co-infusion with bone marrow transplant	Increased survival	[119, 120]
Systemic Lupus Erythrematosus	Rodent MRL/lpr mouse	Intravenous infusion	Recapitulation of bone marrow osteoblastic niche Reduction in autoantibody levels Improvement in kidney function Reduction in ANA Increase in FOXP3+ cells	[121]
Acute Disseminated Encephalomyelitis (Multiple Sclerosis)	Rodent Experimental Autoimmune Encephalomyelitis	Intravenous infusion	Improved clinical score Reduced demyelination Reduced immune cell infiltrate into CNS	[122-124]
Pulmonary Fibrosis	Rodent Bleomycin induced lung injury	Intravenous infusion	Reduced inflammation Reduced collagen deposition Reduced MMP activation	[101, 102]

[a]Abbreviations: ANA, anti-nuclear antigen; CNS, central nervous system; LAD, left anterior descending; NOD, nonobese diabetic; HLA, human leukocyte antigen; MMP, matrix metalloproteinase.

In addition to promoting tissue repair directly, MSCs have also been shown to modulate the immune system and attenuate tissue damage caused by excessive inflammation. Initial indications regarding the immunomodulatory aspects of MSCs were first observed in the context of MSC transplantation studies in animals and humans. Unexpectedly, MSCs seemed to exhibit an unusual ability to evade the immune system. Initial clinical trials showed that autologous and allogeneic MSCs could be transplanted without immune rejection [35, 37]. Further preclinical studies presented similar findings: Human MSCs can engraft and persist in many tissues in prenatal and adult sheep with no apparent rejection [125]; MSC injection in baboons can prolong the life of a transplanted skin graft and suppress T cell proliferation in a dose-dependent manner [126]; and injected MSCs can suppress the immune response in mice, allowing for the expansion of tumor cells [127]. The immunosuppressive ability was first exploited clinically in the treatment of an 8-year-old boy with severe, acute GvHD, who was refractory to steroid immunosuppression [128]. The patient was successfully treated by MSC transplantation. In recent years, this immunosuppression has been found to be an active process, and the mechanisms underlying MSC immunomodulation operate at different levels of the innate and adaptive immune system.

2.4.2. Decomposing the Interaction between MSCs and the Immune System

Many studies suggest that MSCs can promote the conversion from a T_H1 (cell-mediated) immune response to a T_H2 (humoral) immune response (**Figure 2.3**) [99]. In vitro coculture experiments have been used to exemplify the effects of MSCs on individual populations of immune cells that favor this conversion at the cellular and molecular levels. With respect to adaptive immunity, the majority of in vitro studies have shown that MSCs can directly inhibit CD3+, CD4+ T cell proliferation and secretion of T_H1 lymphokines, such as IL-2 and IFN-γ. These studies have induced T cell activation by various methods including mixed lymphocyte reactions (MLRs), mitogens, and T cell receptor (TCR) or costimulatory receptor engagement. T cells in the presence of MSCs appear to be anergized by the lack

of a second danger signal by MSCs, which do not express the costimulatory molecules CD80, CD86, and CD40 [129]; however, this has yet to be definitively proven.

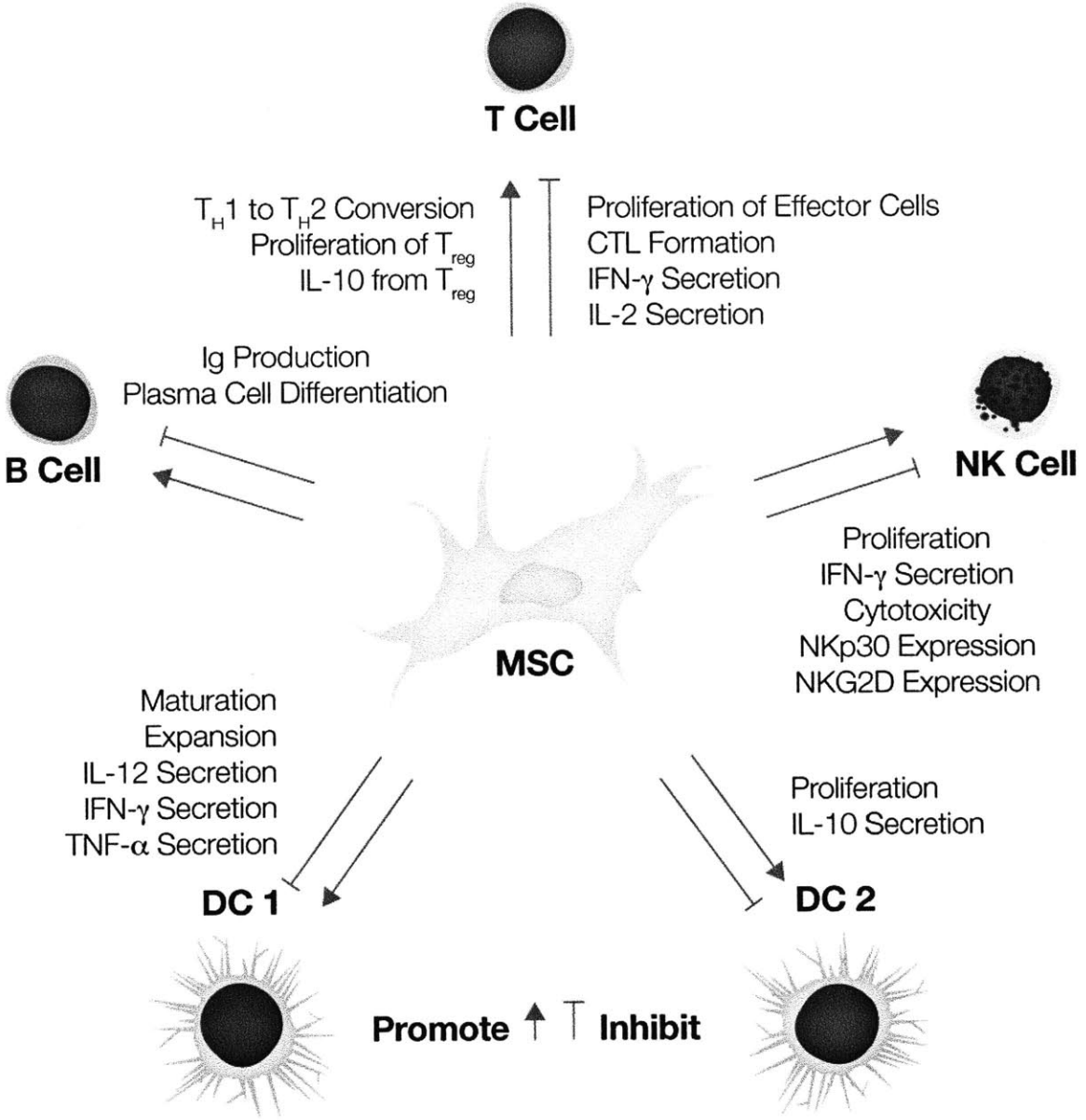


Figure 2.3 – Summary of MSC interactions with select immune cells. Abbreviations: T_H1, helper type 1 T cell; T_H2, helper type 2 T cell; T_{reg}, regulatory T cell; IL, interleukin; CTL, cytotoxic T lymphocyte; IFN-γ, interferon-γ; NK, natural killer; DC 1, type 1 dendritic cell; DC 2, type 2 dendritic cell; TNF-α, tumor necrosis factor-α; Ig, immunoglobulin. Partially adapted from [130].

Several investigations have also shown a direct suppressive effect of MSCs on cytotoxic CD8+ T cells. MSCs prevented cytolysis of target cells by alloantigen-specific CD8+ T cells when present during the priming of cytotoxic cells [131]. Some investigators attribute the inhibition of cytotoxicity by MSCs to an intrinsic "veto" function or to the generation of suppressor CD8+ cells after coculture [132], although conflicting data exist. Other reports have also observed generation of CD4+ CD25+ T cells, a cell-surface-marker expression pattern of both newly activated CD4+ lymphocytes and regulatory T cells [133]. Although it is unclear whether MSCs directly influence B cells in vivo, some in vitro evidence suggests that MSCs can suppress B cell proliferation [134, 135]. In contrast, other reports have shown that MSCs can stimulate antibody secretion and induce polyclonal differentiation and expansion of healthy human B cells [136, 137], consistent with the supportive role of stromal cells in B lymphopoiesis. In addition, these same supportive mechanisms may advance the progression of B cell–mediated disease such as multiple myeloma and systemic lupus erythematosus [138]. However, it is possible that suppression of T cells by MSCs may contribute to decreased B cell activity in vivo [139].

The switch from a cell-mediated to humoral immune response triggered by MSC transplantation may involve the differentiation of innate immune cells to an anti-inflammatory phenotype. Within an inflamed tissue environment, MSCs are capable of influencing many aspects of the cytotoxic responses to injury and disease [140]. MSCs can attenuate natural cytotoxic responses of neutrophils by dampening respiratory burst and inhibiting spontaneous apoptosis in vitro via secretion of IL-6 [141]. MSCs also possess the ability to suppress proliferation of natural killer (NK) cells [142-144] and attenuate their cytotoxic activity by downregulating the expression of NKp30 and NKG2D, surface receptors involved in NK cell activation [145]. This is accomplished even while cytokine-activated NK cells are capable of killing MSCs in vitro, suggesting a possible mechanism for MSC rejection in vivo [142]. In vivo studies have yet to be performed to demonstrate this mechanism. MSCs can also revert macrophages to adopt an anti-inflammatory phenotype in the context of sepsis by secreting prostaglandin E2 and conveying a contact-dependent signal to promote IL-10 secretion [146].

Dendritic cells (DCs) serve as the major link between innate and adaptive immunity because of their ability to present antigens to lymphocytes with high efficiency. In coculture with MSCs, monocytes failed to differentiate into DCs when cultured in lineage-specifying growth conditions [147, 148]. In addition, MSCs inhibited the maturation of DCs to present appropriate antigens and costimulation to T cells through CD1a, CD40, CD80, CD86, and HLA-DR [147, 149]. After coculture with MSCs, DCs were ineffective in their ability to activate lymphocytes by suppressing TNF-α and IFN-γ expression and upregulating IL-10 in DC-CD4+ MLRs [130, 148]. This interaction was found to be γ-secretase dependent, indicating the role of the Notch pathway in MSC-DC interactions [150]. Ultimately, MSCs may drive, or "license," DCs to a suppressor phenotype that can further attenuate T cell–mediated immunity.

2.4.3. Potential Side Effects of Mesenchymal Stem Cell Therapy

MSC transplantation has been designated safe by the FDA. Thus far, clinical trials of MSC transplantation have shown no adverse events that affected the safety profile of these cells over the past 10 years of testing. Nevertheless, recent preclinical studies have highlighted potential long-term risks associated with MSC therapy that may not be observable in the short time period following administration (see **Figure 2.4**). These risks include potential maldifferentiation, immunosuppression, and instigation of malignant tumor growth.

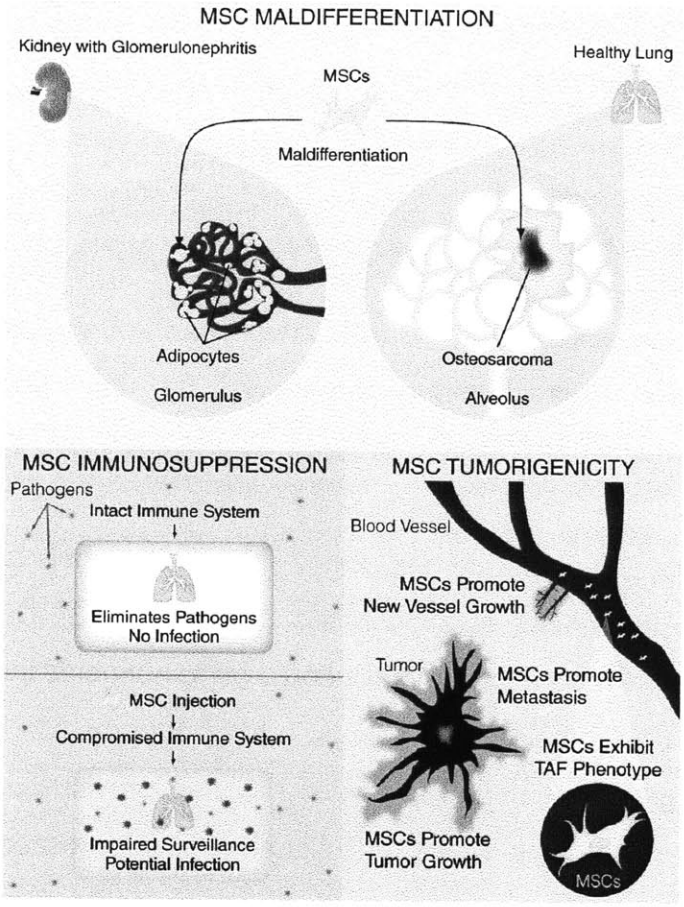


Figure 2.4 – Potential risks associated with MSC transplantation. (*a*) MSCs have been shown to maldifferentiate into glomerular adipocytes and osteosarcomas when administered systemically. (*b*) Systemic administration of MSCs may impair immune surveillance, making the recipient more susceptible to opportunistic infections. (*c*) When transplanted with cancer cells, MSCs can adapt a tumor-associated fibroblast phenotype and support the growth of the cancer by directly promoting tumor growth, metastasis, and angiogenesis. Abbreviations: MSC, marrow stromal cell; TAF, tumor-associated fibroblast.

MSCs are multipotent cells and may ectopically differentiate after therapeutic transplantation. Recent animal studies have confirmed that this is possible. MSCs, when administered in the context of acute glomerulonephritis in rats, can engraft in the renal tubules and maldifferentiate into adipocytes that hinder normal function of the kidney and lead to chronic kidney disease [151]. When administered in mice, MSCs can also create microemboli and subsequently form osteosarcoma-like pulmonary lesions [152]. However, this phenomenon has been observed in immunocompromised mice and therefore may not reflect a considerable risk to immunocompetent hosts. A similar observation was made when MSCs were administered to nonobese diabetic (NOD) mice in the context of IDDM [109]. The MSCs formed soft tissue and visceral tumors throughout the mice upon administration. These studies may indicate risks of MSC transplantation that may be of particular importance to immunocompromised patients.

Other potential complications of MSC transplantation are related to the immunosuppressive properties of these cells and the loss of immunosurveillance to foreign and host pathogens. After MSC infusions were used to treat nine patients suffering from GvHD, three developed viral infections [153]. Although these patients were at increased risk for developing opportunistic infections through the nature of their disease, concerns were raised that immunosuppression by the MSCs had caused a reduction of immunosurveillance to viruses.

These findings are supported by in vitro observations that lymphocyte proliferation by herpes viruses is suppressed by MSCs [153]. Cancer is another potential serious side effect. Theoretically, MSCs could be tumorigenic through direct transformation, metabolism of chemotherapeutic agents, and/or suppression of the antitumor immune response. All these phenomena have been reported previously. MSCs have the potential to transform into sarcomas when Wnt signaling is suppressed [154]. Mesenchymal cells have been shown to regulate the response of acute lymphoblastic leukemia to asparaginase chemotherapy by metabolizing the drug via their high expression of asparaginase synthetase [155, 156]. Marrow stromal cells, highly enriched for MSCs, promote survival of B- and plasma-cell malignancies by inducing hedgehog signaling [157]. Moreover, MSCs used for the treatment of GvHD limited the graft-versus-leukemia effect of allogeneic bone marrow transplantation, leading to a higher rate of relapse compared with control groups [158, 159]. Patients with increased risk of malignancy or opportunistic infections may not be suitable candidates for MSC therapy; researchers should take this into account when assessing trials until more conclusive experimental data become available.

MSCs also associate with tumors and promote tumor growth when administered systemically into animals with existing malignancy [160, 161]. Animal studies by Djouad and colleagues have revealed enhanced tumor growth after MSC transplantation when tumor cells were implanted [127, 162]. It is unclear whether the MSCs enhanced tumor growth by immunomodulatory, trophic, or other effects. Karnoub et al. demonstrated that

MSCs within tumor stroma promote breast cancer metastasis via cancer cell–induced de novo secretion of the chemokine CCL5 in MSCs [161]. Recent work has shown that MSCs can differentiate into tumor-associated fibroblasts (TAFs) that provide stromal support to growing tumors [163]. When mixed with tumor cells and transplanted in vivo, MSCs fulfill the following four criteria that are required for them to be considered TAFs: (*a*) expression of fibroblast markers FAP and FSP; (*b*) secretion of neovascularization promoters VEGF, desmin, and α-smooth muscle actin; (*c*) secretion of tissue remodeling and invasion proteins TSP-1, Tn-C and SL-1; and (*d*) secretion of tumor-promoting factors hepatocyte growth factor (HGF), epidermal growth factor (EGF), and IL-6 [163]. It should be noted, however, that the number of MSCs mixed with the cancer cells exceeded a typical systemic dose, and therefore they may represent a risk only if excessive numbers of MSCs are administered. Although MSCs were not specifically demonstrated to arise from the bone marrow in these studies, evidence that MSCs possess the ability to differentiate into TAFs is consistent with other studies showing that mesenchymal stem cells are recruited to the sites of indolent tumors and promote growth [164]. Osteopontin is implicated as one of the key hormonal mediators of this effect, and it has been shown to be secreted by MSCs that have begun to differentiate down the osteoblast lineage [91]. Future studies will be required to demonstrate direct causality.

Interestingly, some investigators are using the "tumor-homing" properties of MSCs for therapeutic use by genetically engineering the cells with cytolytic drugs to kill tumorigenic tissue selectively [165]. MSCs that are genetically engineered to express TRAIL, a ligand for death receptors on the surface of tumor cells, can suppress tumor growth among subcutaneous tumors, pulmonary metastatic tumors, and highly malignant glioblastoma tumors in mice [166, 167]. Also, MSCs engineered to deliver IFN-β are capable of migrating into the brain and providing survival benefit to mice with gliomas [168]. Using the homing properties of MSCs as such may present a new opportunity for use of MSCs as drug delivery vehicles in the context of cancer.

2.5. Molecular Particle Theory of Mesenchymal Stem Cell Therapy

Therapeutic studies in different injury models accompanied by cell-tracking studies have revealed two peculiar observations: First, infusion of undifferentiated MSCs leads to therapeutic effects in different injury models without MSC differentiation. Second, the majority of MSCs cannot be located by sensitive, whole-body imaging techniques days after transplantation. In this section, we discuss studies that suggest that MSCs may impart therapeutic benefit by secreting soluble factors, and then we paint a molecular portrait of MSCs. Next, we highlight particular biodistribution studies that describe the rapid kinetics of MSC clearance after transplantation similar to what might be found when inert particles are injected. Finally, we close with a new proposed framework for pharmacokinetic analysis of MSC therapy.

2.5.1. Molecular View of Mesenchymal Stem Cells

Recent work has shown that the therapeutic benefits observed when MSCs are transplanted can be completely recapitulated, and in some cases improved upon, by administration of MSC secreted factors alone. This is not surprising because suppression of effector functions in most MSC-immune cell coculture studies was reproduced in the absence of cell-cell contact and in a dose-dependent manner, indicating the role of soluble factors. Furthermore, these inhibitory molecules can exert their effects across species barriers as evidenced by suppression of MLRs in xenogeneic cultures [169]. MSC-conditioned supernatants have no antiproliferative effect on T cells, yet they are capable of suppressing the stimulation of B cells [134]. This suggests that MSCs can dynamically react to their immunological environment in the context of T cells while also secreting immunomodulatory molecules in their quiescent, undifferentiated state in the context of B cell development. Also, the numbers of MSCs needed to suppress T cell activity compared with B cell activity differ by approximately one to two orders of magnitude [135, 170]. These

studies hint at the interesting dynamics and dosing of MSC-derived molecules that researchers should consider when evaluating MSC therapeutic applications.

It remains highly debated as to which soluble mediators are involved in MSC therapy (reviewed in Reference [171]), although a clear distinction can be made that some molecules are considered naturally secreted by MSCs and others are inducible. Many candidates such as HGF, transforming growth factor β_1 (TGF-β_1), or the metabolic byproduct of indoleamine 2,3-dioxygenase (IDO) [99, 172-176] are basally secreted by these cells. However, stimulation of MSCs by toll-like receptor ligands or inflammatory cytokines causes an alteration of the MSC secretome and a different set of chemical species [177, 178]. For example, lipopolysaccharide (LPS) found in serum leads to the rapid upregulation of prostaglandin E_2 (PGE_2), likely through an immediate early gene response related to NF-κB. Recently, researchers demonstrated a direct correlation between the upregulation of an anti-inflammatory protein, TSG-6, upon engraftment of MSCs in the lungs and the recovery of myocardial function after infarction [14].

In our laboratory, we have sought to leverage this collection of bioactive molecules to treat animals undergoing inflammatory organ injury. We have developed methods for intravenously administering MSC-derived molecules in the form of concentrated conditioned medium, as well as in a dynamic and continuous manner by using an MSC extracorporeal bioreactor. Initially, it was our intent to treat rats undergoing D-galactosamine-mediated organ injury by transplanting human MSCs. However, upon transplantation of the cells, we observed no benefit to the animals as measured by 7-day survival [41]. In contrast, by systemically administering the equivalent mass of lysed cells, we observed a survival trend, suggesting that the molecules relinquished upon lysis provide the primary benefit. We confirmed this hypothesis by collecting the molecules secreted in MSC conditioned medium (MSC-CM) and administering a concentrated form of MSC-CM intravenously in a single bolus dose to animals after the induction of disease. We found that MSC-CM provides a statistically significant survival benefit, causing an increase in survival from 14% in our control groups to 50% in our treatment group [42]. We then engineered a

delivery platform for the continuous and dynamic administration of these molecules into the bloodstream [41] and found that this treatment method provided an improved survival benefit of 71% compared with 14% in controls.

These studies collectively suggest that the soluble factors of MSCs account for the majority of beneficial effects in response to MSC transplantation. Considering the putative natural functions of MSCs, it is reasonable to postulate that the same secretory mechanisms by which MSCs maintain hematopoiesis are therapeutic in the context of disease. In fact, many of the MSC factors that have been implicated in hematopoiesis have also been shown to provide therapeutic benefit in certain disease models. Many insist that upon transplantation of the cells, engraftment, proliferation, differentiation, and homing to the site of injury are equally important. However, new evidence is suggesting that MSC engraftment rarely occurs, and observed therapeutic benefits of MSC transplantation may arise from other alternative explanations.

2.5.2. Particle View of Mesenchymal Stem Cells

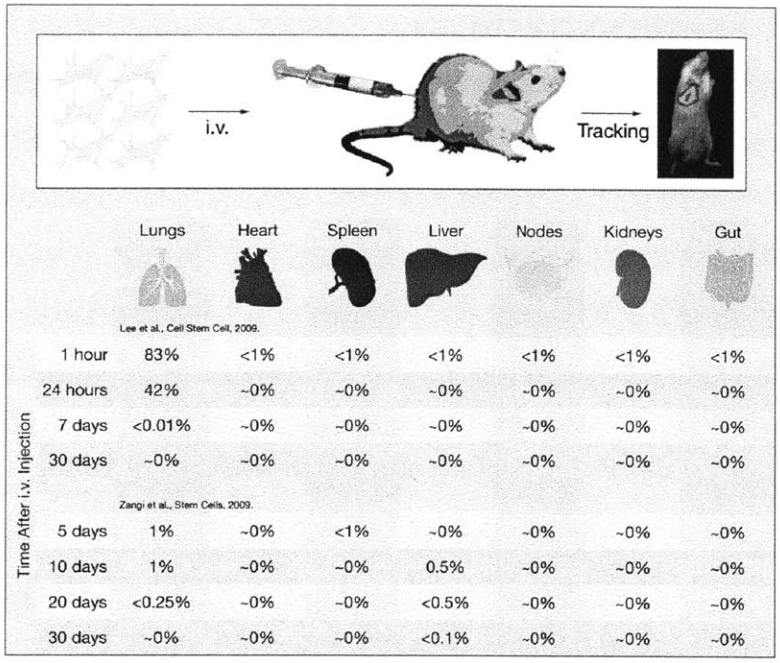
Early qualitative studies using MSCs stably transfected with a fluorescent reporter gene for bone regeneration revealed interesting dynamics of the MSC grafts in vivo. First, engraftment of osteoprogenitor cells was found to be saturated, suggesting that higher doses of cells would be an ineffective strategy to improve engraftment [179]. Second, temporal tracking of enhanced green fluorescent protein (eGFP)-expressing MSCs showed that transplanted cells exhibited limited proliferation and self-renewal capacity after engraftment, yet they could be serially passaged and repopulate another host [180]. Similar studies have evaluated different reporter strategies, but most techniques used are not amenable to whole-subject imaging by nature and thereby may be confounded by selective tissue sampling.

Quantitative examination of the biodistribution of MSCs within the body after transplantation suggests that the dynamics of an MSC graft are similar to that of inert

micrometer-scale particles injected into the blood stream of animals [181]. Recent studies using sensitive cell-tracking methods have revealed these counterintuitive results (**Figure 2.5**). Most homing and engraftment studies have demonstrated little, if any, long-term engraftment (>1 week) of MSCs upon systemic administration. Studies have shown that the majority of administered MSCs (>80%) accumulate immediately in the lungs and are cleared with a half-life of 24 h [14, 182, 183]. Tissue-specific homing has been demonstrated, indicating a response of the administered MSCs to injured tissue [184]. It was shown in a mouse model of myocardial infarction that MSCs are capable of engrafting in the site of injury and differentiating into cardiomyocyte-like cells that were shown via immunohistochemistry to express typical cardiomyocyte markers [185]. Another study showed that in mice undergoing cisplatin-mediated acute kidney injury, systemic injection of MSCs resulted in accumulation of MSCs in the kidney and differentiation into tubular epithelial cells that exhibited the characteristic brush border of the proximal tubule [186]. Nevertheless, the majority of studies have shown that only a small percentage of the original systemically administered cell mass is capable of engrafting even under the best conditions, and of those that do engraft, only a small percentage have been shown to differentiate into functional replacement tissue.

These homing, engraftment, and differentiation studies illustrate one of the most persistent paradoxes in MSC therapy: Systemically administered MSCs appear to convey potent therapeutic responses in a variety of diseases, and yet they have not been shown to exhibit long-term engraftment. Adding further complexity, recent studies have suggested that allogeneic MSCs are not immunoprivileged, that they enjoy a only minimally prolonged residence time compared with allogeneic fibroblasts [15], and that they may elicit a memory response leading to a rapid clearance of subsequent doses by the immune system. It is not unreasonable to suggest, therefore, that instead of engrafting and differentiating, MSCs convey therapeutic benefit by relinquishing their molecular contents. In this way, MSCs may be better viewed as drug delivery particles that, when administered, are subject to distribution and clearance similar to other intravenous therapeutics. Therefore, if the cells are to be delivered optimally, quantitative analysis regarding their pharmacokinetics will

better inform dosing regimens to account for the limits and advantages of particle drug delivery as such.



Time After i.v. Injection	Lungs	Heart	Spleen	Liver	Nodes	Kidneys	Gut
	Lee et al., Cell Stem Cell, 2009.						
1 hour	83%	<1%	<1%	<1%	<1%	<1%	<1%
24 hours	42%	~0%	~0%	~0%	~0%	~0%	~0%
7 days	<0.01%	~0%	~0%	~0%	~0%	~0%	~0%
30 days	~0%	~0%	~0%	~0%	~0%	~0%	~0%
	Zangi et al., Stem Cells, 2009.						
5 days	1%	~0%	<1%	~0%	~0%	~0%	~0%
10 days	1%	~0%	~0%	0.5%	~0%	~0%	~0%
20 days	<0.25%	~0%	~0%	<0.5%	~0%	~0%	~0%
30 days	~0%	~0%	~0%	<0.1%	~0%	~0%	~0%

Figure 2.5 – Representative studies describing the in vivo distribution of MSCs upon systemic administration. Tracking studies generally consist of intravenous injection of the cells and then tracking of the cells using a variety of known methods. The representative studies featured here used two sensitive methods available for whole-organism analysis: polymerase chain reaction of a human gene to quantify human MSC engraftment in a number of mouse tissues, and MSCs labeled with luciferase to qualitatively trace their engraftment. Abbreviation: iv, intravenous.

2.5.3. The Pharmacokinetics of a Mesenchymal Stem Cell Graft

A reductionist view of MSCs as particles loaded with a molecular drug may enable efforts to predict the efficacy of this cellular therapeutic within the confines of known physical models of drug delivery systems. By extracting quantitative parameters from retrospective studies, we plan to take a mathematical approach to propose designs of new formulations and dosing regimens composed of MSC therapy. The objective of this exercise is to maximize the therapeutic activity of MSCs while minimizing potential side effects associated with MSC transplantation such as maldifferentiation and tumor growth.

As with most physical models, we make a few basic assumptions and approximations to simplify the problem in order to display analytical solutions. It would perhaps be more fitting to use computational approaches to solve the complex set of partial differential equations that form the basis of this theory, but doing so would exceed the scope of this initial model. We begin by modeling MSCs as inert, spherical (d = 20 μm) particles that have no interactions with host structures. Furthermore, we assume that a single molecule of fixed concentration is encapsulated within each MSC and contains 100% of the bioactivity. The transport of this single molecule from the cell directly into the bloodstream is not rate limiting. Finally, we assume that a defined therapeutic index exists with respect to the single molecule that directly correlates to serum concentration profile.

With these assumptions in place, we first consider the cell mass used in clinical studies and employ order-of-magnitude approximations to determine if the current clinical dosing of MSCs is justified. If cells secrete ~100 pg 10^{-6} cells per day of a therapeutic mediator, which corresponds to ~0.1 fg per cell (assuming the same intracellular levels of the mediator), then we can estimate that when a clinical-scale mass of MSCs (~100 × 10^6 cells) is infused intravenously, this equates to ~10 ng of a therapeutic molecule. In comparison with other biologics, which are administered in the microgram to milligram range, we can immediately see that the clinically administered dose of molecules relinquished from MSCs is at least —two to three orders of magnitude lower than that of other single-molecule therapeutics. This simple analysis would suggest that a greater cell mass should be administered, but it does, of course, grossly underestimate the biological complexity of the molecular mixture composed within MSCs, which is multifactorial in nature and may have synergistic effects.

With respect to the temporal dosing of an MSC graft, we have taken known kinetics data concerning MSC transplantation from selected studies [14, 15] and have represented these data in a new form to illustrate this concept graphically (**Figure 2.6**). In the extreme case, and perhaps consistent with early notions, effective MSC transplantation assumed that nearly 100% of the cells remain viable and active after infusion into a subject. Therefore,

the units of activity of the transplant would approach steady state in the timescale of days to weeks after administration. However, if we plot the normalized cell concentration found in tissues within 1–120 h based on previous studies, we see that the kinetics of an MSC graft is much more transient than expected with a half-life of approximately 24 h. Using a two-compartment pharmacokinetic model, we can extract parameters that explain the discrepancy between theoretical and apparent bioavailability of MSCs. Assuming an intravenous bolus where the infusion rate (R_i) is eliminated and the dimensionless plasma concentration $C_p(t = 0)$ is equal to 1, we find that the rate of tissue intravasation (K_2) and the rate of clearance (R_c) are significantly greater than the theorized tissue extravasation (K_1), resulting in a much shorter half-life of the cellular therapeutic. Furthermore, we see that the apparent activity of the treatment, represented as the time to reach maximal secretion of a molecular mediator, is extremely sensitive to the cellular viability and reinforces the concept of a short therapeutic window associated with MSC therapy. If we arbitrarily choose a minimum effective concentration of MSC therapy and transpose this timescale to a biological response, we see that transplanted MSCs are only therapeutically active for a short period of time (in this case, less than 24 h). This timescale corresponds precisely with measured serum cytokine levels that are directly associated with MSC therapy [146] and that potentially can be considered surrogate biomarkers for effective therapy. Ultimately, successive doses of MSCs within a shorter treatment period may allow for the maintenance of MSC therapy within a therapeutic window that can ultimately sustain a long-term biological response.

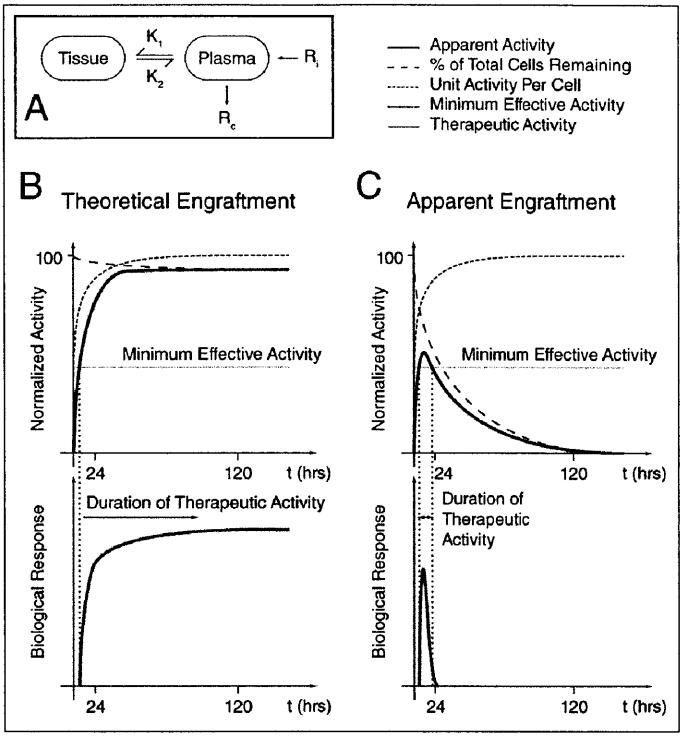


Figure 2.6 – Pharmacokinetic analysis of MSC therapy. (*a*) Schematic of two-compartment pharmacokinetic model of MSC drug delivery incorporating the following parameters: R_i, injection rate; R_c, clearance rate; K_1, rate of extravasation; K_2, rate of intravasation. (*b*) Theoretical engraftment of MSCs with a hypothetical retention of nearly 100% of MSCs over the course of 120 h. The apparent activity is the product of the unit activity per cell and the number of cells remaining after injection. Assuming a minimum effective activity level well below the apparent activity, the biological response would be expected to rise as soon as the minimum effective activity level is reached and to be sustained thereafter. (*c*) Apparent engraftment of MSCs with a decaying retention of MSCs. Assuming an exponential decay with a 24-h half-life, the apparent activity peaks above the minimum effective activity level only for a brief period of time. This results in a brief and temporary biological response that does not persist beyond 24 h. These data are consistent with cytokine response associated with MSC transplantation or MSC-derived molecules when the latter were administered to animals undergoing systemic inflammation.

2.6. Conclusions

Developing new therapies that affect multiple disease pathways is of growing importance for patient care. MSC transplantation represents an exciting approach that could potentially treat complex diseases by providing combinatorial therapy. Furthermore, the continued use of MSC therapy can be recast to better our understanding of the natural role of these cells during health and disease in vivo. The collected efforts of scientists, engineers, physicians, and industry will be necessary to realize the promise of MSC therapy.

Optimization of MSC therapy may not be achievable until the primary mechanism(s) of action afforded by intravenous administration of MSCs is determined. Current optimization

approaches are based on the number of MSCs used, but this parameter alone may not capture the true activity of this complex therapy. The therapeutic activity of an MSC graft was once thought to be a function of engraftment and differentiation. However, mounting evidence is indicating that MSCs can impart activity independent of these functions. Trafficking studies have reported that systemically administered MSCs fail to engraft in most tissues and that engrafted cells may eventually be rejected, resulting in immunological memory to subsequent treatments [14, 15]. This new evidence suggests that the activity of MSC formulations may have half-lives on the order of an inert particle. This timescale should be considered when new dosing regimens for MSC therapy are being defined.

To simplify the explanation of cellular biodistribution, we have considered MSCs as inert particles. However, in reality they are by no means inert. Bone marrow MSCs actively participate in the maintenance of hematopoiesis and therefore influence the development of cells in the immune system. MSCs can differentiate into stromal lineages that provide the cellular and structural elements required to support hematopoiesis. Beyond cellular differentiation, MSCs fortify the chemical milieu of the bone marrow by secreting many immunoregulatory and trophic factors that contribute to the successful development of the blood cells. In essence, MSCs are intimately associated with homeostatic mechanisms that tightly regulate activity in the bone marrow microenvironment. As a consequence, MSCs may be naturally equipped to interact directly with the immune system and influence it by mechanisms that are concordant with the origins of MSCs. Indeed, the natural target tissue of MSC therapy may very well be the hematopoietic system.

By using previous examples of molecular therapeutics as an initial framework, we have attempted to describe the theoretical behavior of MSCs upon administration in order to identify some options to consider when developing dosing regimens. Using our simplified model, we can glean that cell therapy may need to be administered at a greater magnitude and/or frequency to sustain a long-term biological response. Moreover, methods that improve the half-life of the graft in vivo by increasing cell survival and engraftment or by decreasing cell clearance may also be viable options to enhance therapeutic activity.

Furthermore, this model underscores the need to identify these mechanisms that govern cell fate in vivo as well as practical and relevant biomarkers that can be used to monitor the activity of MSCs after administration.

MSCs have the potential to treat many unmet medical conditions that afflict patients every day in a manner that is consistent with the human body's natural capacity to heal itself. Development of quantitative means for harnessing the source of MSC therapeutic activity will therefore ensure we make the most of everything these cells can accomplish.

3. Leveraging Mesenchymal Stem Cell Secretions for Therapy

3.1. Introduction

In the last chapter, we reviewed the state-of-the-art of MSC therapeutics, from *in vitro* findings, animal models of disease, and MSC transplantation in human subjects. In the final sections of the chapter we began to hint at the limitations in the field currently in terms of a lack of understanding of the mechanisms of action of MSC transplantation, especially as it pertains to the pharmacokinetics of MSC grafts. In this chapter we will describe our work with MSC secreted factors, which we have found to exhibit potency and efficacy comparable to, and in some cases superior to MSC transplantation. We will begin by presenting the results of our studies wherein we tested the therapeutic benefit of MSC conditioned medium (MSC-CM) to treat rats with acute kidney injury (AKI). Then, as the next step towards translating the delivery platform for MSC secreted factors into clinical use, we will describe our work in developing a clinical-scale bioreactor for use in large animals and patients with AKI.

Before we begin, however, I want to introduce various concepts that will be relevant to the results reported, with a particular focus on acute kidney injury.

3.1.1. Etiology, Clinical Definition and Pathophysiology of Acute Kidney Injury

Acute kidney injury (AKI) is a devastating syndrome that accounts for hundreds of thousands of deaths annually worldwide [187]. Despite advances in patient management and renal support therapy technologies, this devastating mortality has not significantly changed in the last 50 years; even with the best treatment available, the mortality associated with AKI is as high as 70% among patients receiving dialysis. Generally

characterized by a reduction in glomerular filtration rate (GFR), AKI has many root causes **(Table 3.1.1)** [188-196]. The most common cause of AKI is renal ischemia, occurring in over 50% of patients with AKI.

Table 3.1.1 – Causes of AKI and the Pathological Manifestations

Causes of AKI	***Incidence***	***Pathological Manifestation***
Hypovolemia	*~60%*	*Necrosis, Apoptosis of Tubules*
Vascular occlusion		*Necrosis, Apoptosis of Tubules*
Sepsis		*Necrosis, Apoptosis of Tubules, Nephritis*
Drug (radiocontrasts, microbials, chemotherapeutics)		*Necrosis, Apoptosis of Tubules*
Hepatic dysfunction		*Nephritis*
Primary renal disease	*~25-35%*	*Necrosis, Apoptosis of Tubules, Nephritis*
Impaired renal perfusion		*Necrosis, Apoptosis of Tubules*
Obstruction of the collecting system	*~5-15%*	*Necrosis, Apoptosis of Tubules*

The clinical definition of AKI varies. In general, AKI is characterized by an abrupt loss of renal function, resulting in electrolyte misbalance, failed waste excretion, an inability to concentrate urine, and a loss of fluid regulation [190]. Specifically, AKI often is associated with a reduction in renal blood flow from increased renal vascular resistance, a decrease in the ultrafiltration coefficient, and/or tubular obstruction [197].

Because the etiology of AKI is varied, the complete pathophysiology of AKI is case-dependent. It is thought that there are nearly 50 different pathophysiological pathways that describe the onset of AKI [192]. Nevertheless, most instances of AKI share certain hallmarks that are characteristic of the disease regardless of cause. Generally, AKI entails the rapid decline of kidney function over the course of hours to days. This is often the result of either: (1) a significant decrease in renal perfusion; or (2) toxic damage to the parenchyma of the organ. In the case of decreased renal perfusion, hypoxia inhibits oxidative phosphorylation, depleting the organ of energy, and induces a pro-inflammatory state in the organ by stimulating the vascular endothelium and resident leukocytes [198, 199]. Also, depending on the perfusion state, low renal perfusion can activate the renin-

angiotensin system (RAS), resulting in vasoconstriction of the afferent and efferent arterioles, exacerbating the impaired blood flow. The resultant ischemia leads to a loss of function in the tubule epithelia, precipitating failure to properly maintain electrolyte homeostasis, fluid balance, blood pH, and excretion of metabolic wastes, in addition to further activation of the epithelium, endothelium and resident leukocytes. A combination of impaired oxygen delivery; accumulation of metabolic wastes; and up-regulation of inflammatory cytokines, reactive oxygen species (ROS), and endothelial adhesion molecules contribute ultimately to leukocytic and lymphocytic infiltration and tissue death [200-202]. In the case of toxic damage, foreign substances in the blood cause similar tissue death as in the case of ischemia [203]. In both instances, the hypoxic activation of endothelium, epithelium and resident leukocytes, in addition to the growing presence of dead and dying tissue further intensify the pro-inflammatory state, causing continued release of ROS, transmigration of neutrophils, cytokine release, extravasation, edema, and irreversible tissue damage [204]. **Figure 3.1.1** summarizes the pathophysiology of AKI.

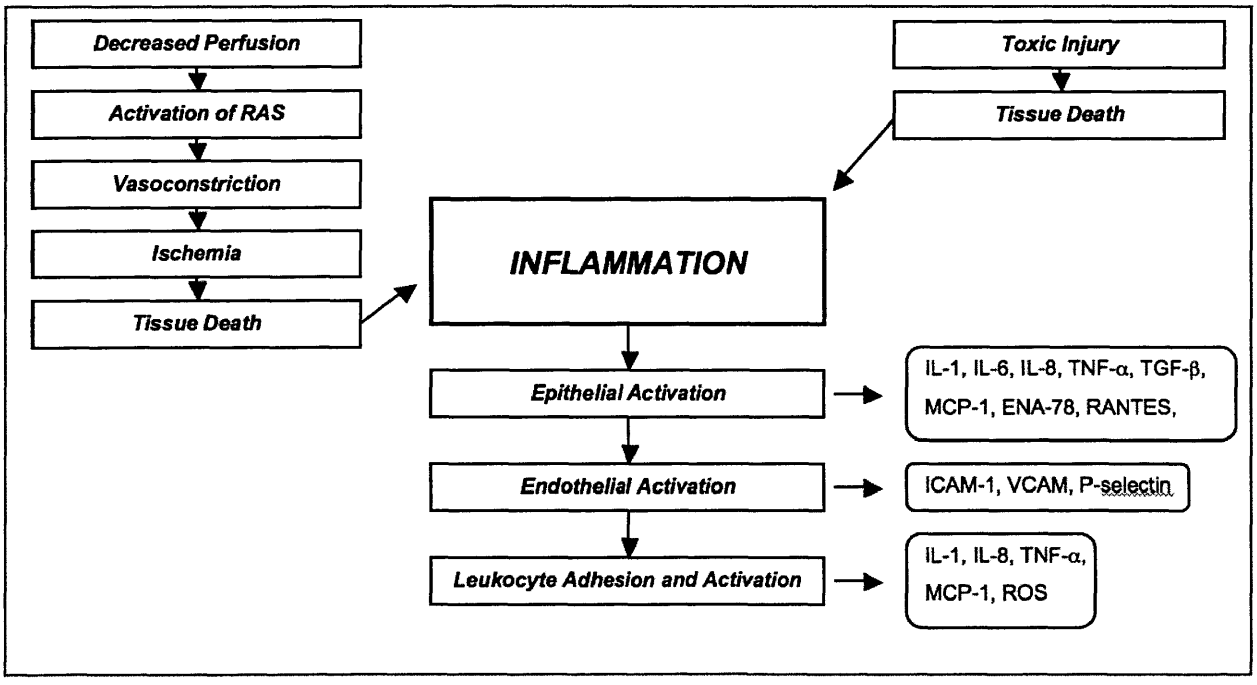


Figure 3.1.1 – Pathophysiological hallmarks of AKI.

Inflammation is thought to be at the root of the devastating mortality and resistance to treatment among patients with AKI [200, 205, 206]. The onset of AKI is not necessarily immune-mediated, but as soon as the kidney is damaged due to ischemia or toxic injury, inflammatory processes greatly exacerbate the insult to the organ. Ultimately, it is this inflammatory insult that causes irreversible damage to occur, greatly impairing the natural functions of the kidneys, and greatly increasing the probability of death. Novel approaches, therefore, are currently under development that aim to attenuate the inflammation underlying AKI. These approaches have thus far shown more promise than former treatment modalities involving replacement of primary renal function.

3.1.2. Current Extracorporeal Treatment Options and Trials in Cell-Based Renal Support Devices

For patients undergoing AKI, there are standard treatment options available depending on the pathophysiology and severity of the disease [207]. In general, patients undergoing AKI are too hemodynamically unstable to undergo organ transplantation or surgery, and standard care is directed at supplementing or replacing lost renal function in order to reestablish homeostasis. The most common supplemental treatment involves administration of fluids to restore volume and vascular tone. To partially replace the function that is lost, renal support therapies (RST) are available. Typical RST treatment involves either intermittent hemodialysis (IHD) or continuous veno-venous hemodialysis (CVVH) [208-211].

New concepts in RST have recently been explored that more completely recapitulate the function of the kidneys to provide dynamic biologic support that is impossible to achieve using passive dialysis [212, 213]. To date, the renal assist device (RAD), developed by David Humes and others at the University of Michigan, has been the most widely tested, and is the only experimental active RST device that has been tried in the clinic [214-221]. The RAD consists of primary human tubular epithelial cells seeded and grown in the intraluminal space of standard polysulfone dialyzers. The device is then connected to a

standard dialysis circuit, and patients are treated with CVVH in series with RAD. Early studies by Humes and Mackay demonstrate the capacity of the epithelial cells in their RAD to actively reabsorb fluid, bicarbonate and glucose; secrete PAH; transport and degrade glutathione; generate ammonia; and activate vitamin D [214, 220]. Further validation studies demonstrated the effectiveness of the device to: (1) support and reverse uremia in dogs [214, 218]; (2) treat 10 patients in the ICU undergoing AKI along with other inflammatory co-morbidities with a survival rate of 60% (compared to 85% expected mortality) [219]; and (3) improve survival from 39% in the control passive CVVH group to 67% in the group of patients treated with CVVH plus RAD [221]. Unfortunately, the use of the RAD was terminated during the Phase IIB trial because no demonstrable survival benefit was observed in larger patient cohorts using the RAD compared to a control device. There was instead a considerable increase in the risk to patients undergoing RAD therapy.

3.1.3. Clinical and Experimental Testing of Immunomodulation Therapy in AKI

It is postulated that the failure of the RAD was due in part to inadequate attenuation of the severe inflammation that is at the root of AKI. New studies increasingly stress the importance of inflammation in AKI, and emphasize the necessity of anti-inflammatory therapies to reverse AKI [222, 223]. In an extreme case of AKI in the presence of paraquat poisoning (serum creatinine of 11.9 mg/dL), researchers reported the reversal of AKI with repeated methylprednisolone pulse therapy and prolonged dexamethasone treatment [224]. Several studies have been conducted to evaluate anti-inflammatory treatment for patients undergoing sepsis, and sepsis-induced multiple organ failure (MOF), including AKI. A number of investigators have evaluated the effectiveness of cytokine antagonists, such as recombinant human interleukin (IL)-1 receptor antagonist (IL-1ra) [225-227], TNF-receptor blockers, or soluble TNF receptors [228]-[229] in patients undergoing sepsis. Although no survival benefit in sepsis was observed, stratification of the trials showed a significant reduction in the cases of shock, which typically precipitates AKI. In a study from 2004, it was shown that the inflammatory profile of patients undergoing AKI could be used

as a predictor of mortality [222]. Specifically, the serum levels of pro-inflammatory molecules IL-1β, IL-6, IL-8, c-reactive protein, and TNF-α were measured, in addition to the anti-inflammatory marker IL-10. During AKI, all cytokines were found to be at a higher level than baseline. Specifically, it was found IL-6 and IL-8 levels were significantly higher in non-survivors and the relative levels of these two cytokines could be used to predict mortality. Interestingly, IL-10 was also found to be significantly elevated in the group of non-survivors, suggesting a complicated inflammatory state that is neither purely pro- nor anti-inflammatory. These human studies suggest the need for a therapy for AKI and septic shock that is dynamic in its response to the inflammatory state of the patient, providing for immune-regulation more so than promotion or inhibition of inflammation.

Animal studies of AKI have revealed the critical role of various aspects of inflammation in the pathogenesis of the syndrome, further suggesting a complex inflammatory state as the underlying cause of high mortality in patients with AKI. In mice deficient of caspase-1, the protease responsible for cleaving precursors of IL-1β and IL-18, the damaging effects of AKI were significantly diminished as measured by a decrease in BUN, serum creatinine, and morphological tubular necrosis score. These findings suggest a significant contribution to AKI pathophysiology by the pro-inflammatory cytokines IL-1β and IL-18 [230, 231]. While it has been shown that knocking out IL-1β alone does not provide for sufficient renoprotection in the presence of AKI, inhibition of IL-1β and IL-18 together could lead to attenuation and potential resolution, suggesting the role of a multi-factorial inflammatory state, and not single inflammatory cytokines as the underlying basis of AKI pathophysiology [232, 233]. Further evidence of such a multi-factorial state was uncovered in studies involving the contributions of endothelial adhesion molecules during ischemic AKI. Kelly et al. demonstrated the effectiveness of a monoclonal antibody (mAb) directed against ICAM-1, a ligand that binds to the β_2 integrins CD11a/CD18 and CD11b/CD18 expressed on the surface of leukocytes, to reduce the damage caused by ischemic AKI in rats [234]. A follow-up study by Linas et al. confirmed the adhesion of leukocytes, specifically neutrophils, via the ICAM-1 ligand in an isolated perfusion model of ischemic AKI in rat [235]. Another study by Kelly et al. showed renoprotection during ischemic AKI in

both ICAM-1 knockout mice, and in mice depleted of neutrophils [236]. Their study yielded no additional benefit to neutrophil-depleted mice administered ICAM-1 mAb, suggesting the specific role of ICAM-1 in the adhesion of neutrophils during ischemic AKI. More recent work has begun to uncover the role of T-cells in the progression of ischemic AKI. In a knockout model of CD4/CD8 in mice, the inhibited T-cell function was implicated to contribute to attenuated organ dysfunction in ischemic AKI [237]. T-cell deficient mice (*nu/nu*) were also found to be protected from injury during ischemic AKI [238]. In this study, CD4 T-cells, CD8 T-cells, and a combination were administered to the knockout mice, and only those groups with reconstituted CD4 T-cells showed renoprotection, suggesting a pivotal role of CD4+ T-cells in ischemia-mediated renal injury.

Taken together, these animal and human studies provide compelling evidence of a complex inflammatory state underlying the pathogenesis of AKI. In order to effectively treat the disease, future therapies will need to address this complex state, and provide for multi-factorial dynamic support.

3.1.4. Attenuating Inflammation During AKI with Marrow-Derived Mesenchymal Stem Cells

Circulating cytokines present during inflammation often causes the bone marrow to mobilize cells to track inflammatory signals and provide immunological support [239, 240]. In general, the cells that are mobilized are pro-inflammatory by nature, and mediate the resolution of infection or damaged tissue by combating pathogens and clearing dead tissue. Only certain cells that are mobilized are capable of attenuating excessive inflammation and promoting tissue repair [241, 242]. In the pathogenesis of inflammatory diseases such as AKI, it has been suggested that a deficit of regulatory cells precipitates an inappropriate immune response to the initial insult [243].

Bone marrow-derived mesenchymal stem cells (MSCs) and their stromal progeny are implicated in the regulatory pathway of injury resolution as they possess the capacity to

modulate inflammation and initiate tissue repair programs [41, 244]. Initial studies investigated the role of the bone marrow in the resolution of AKI [110, 243, 245-248], with more recent studies focusing specifically on the role of MSCs. In 2004, Morigi et al. were the first to demonstrate that among the cells from the bone marrow contributing to repair of the kidneys following cisplatin administration, it was the MSC fraction that was solely responsible for resolving AKI [186]. They went on to assert the MSCs were likely providing regenerative support to the kidney in the form of differentiation into renal tubule epithelium, as evidenced by the detection of Y-chromosome MSCs localized in the renal tubule epithelium of female mice. In 2005, Togel et al. presented evidence of an anti-inflammatory contribution that the MSCs were making to aid in the reversal of AKI [104]. They measured the amount of inflammation afflicting the kidneys during ischemic AKI, and found in mice administered MSCs, gene expression of IL-1β, TNF-α, and IFN-γ was down-regulated compared to mice administered a saline control. Semedo et al. in 2007 confirmed this work with another analysis of the attenuation of inflammation with MSCs administered in rats undergoing AKI [249]. Tögel et al. followed-up their 2005 study with a mechanistic exploration into the role of paracrine factors in the resolution of AKI [105]. They found the MSCs secrete renotropic and vasculotropic factors that aid in the repair and regeneration of the endothelium. More recently, Morigi et al. provided the first evidence that human MSCs were capable of providing the same renotropic and renoprotective support to a cisplatinum model of AKI in mice [112].

Controversy exists concerning the primary role of MSCs in resolving AKI. Proponents of the stem cell hypothesis assert that MSCs are capable of differentiating into cells that aid in the repair of the kidney, specifically endothelial cells and tubular epithelial cells [186]. Recent evidence suggests, however, that the MSCs do not differentiate into endothelium or epithelium in the context of kidney damage, and hence their renoprotective role derives from another mechanism [151, 250, 251].

3.1.5. Summary

The onslaught of uncontrolled inflammation during AKI is thought to be at the heart of the high mortality of the disease. Novel therapies are being proposed that attenuate the inflammation, and provide for organ recovery and tissue repair. Recent work in the realm of MSC-based therapies has indicated a promising role that these cells might play in modulating the inflammation during AKI, providing renoprotection to significantly increase survival. Through paracrine mechanisms, MSCs provide tropic support that is particularly potent in the kidneys. Administration of these paracrine factors, therefore, may provide for a novel therapeutic modality that has yet to be fully explored.

3.2. Secreted Factors from Mesenchymal Stem Cells Upregulate IL-10 and Reverse Acute Kidney Injury in Rats

3.2.1. Abstract

Acute kidney injury is a devastating syndrome that afflicts over 2,000,000 people in the US per year, with an associated mortality of greater than 70% in severe cases requiring dialysis. AKI represents a significant unmet medical need because the standard-of-care treatments are not sufficient for modifying the course of disease; gold-standard therapies are palliative at best. Many groups have explored the use of mesenchymal stem cells (MSCs) for the treatment of AKI because MSCs have been shown to possess unique anti-inflammatory, cytoprotective and regenerative properties *in vitro* and *in vivo*. Thus far, it is yet unresolved whether the primary mechanisms controlling MSC therapy in AKI depend on direct cell transplantation, or whether MSC secreted factors alone are sufficient for resolving the disease. Here we show that MSC secreted factors, independent of cell transplantation, are capable of providing a survival benefit to rats undergoing cisplatin-induced AKI. We observed that when MSC conditioned medium (MSC-CM) is administered intravenously it protects the architecture of the kidney, prevents tubular

apoptosis and necrosis, and reverses AKI as measured by serum BUN and creatinine. In addition, we observed that MSC-CM causes IL-10 upregulation in treated animals, and is important to animal survival. In all, these results demonstrate that MSC secreted factors are capable of providing support at a level similar to whole cell transplantation, and the IL-10 increase seen in MSC-CM treated animals correlates with protection of the kidneys and attenuation of severe AKI.

3.2.2. Introduction

Each year, acute kidney injury (AKI) takes the lives of hundreds of thousands of people worldwide with a likelihood of death greater than 70% in cases of dialysis dependence [187, 190]. AKI is an inflammatory organ failure syndrome that is caused by extensive injury to the kidney parenchyma [252]. There are various etiologies of AKI that stem from pre-renal, intrinsic renal, or post-renal sources. Regardless of etiology, however, it is thought that at the root of the devastating mortality is persistent systemic inflammation that prevents tissue regeneration and disease reversal [223]. Resolution is thought to be achievable if the inflammatory cytokine storm created by AKI can be attenuated, and support is provided to aid in the healing of the injured tissue [253]. Unfortunately, the only existing therapies available to patients with AKI are palliative. Dialysis, while useful to restore electrolyte balance and remove waste products in the blood, weakly supplements the failing kidneys without providing anti-inflammatory or regenerative support [189]. Single molecule therapies directed against AKI have thus far proven insufficient for addressing the complex pathophysiology underlying the disease [254]. New therapies that can resolve the disease by addressing multiple aspects of AKI pathophysiology in parallel are desperately needed.

Mesenchymal stem cells (MSCs) have been explored in pre-clinical and clinical studies as a potential therapeutic for patients with AKI [255], as MSCs have been shown to possess potent immunomodulatory and regenerative properties *in vitro* and *in vivo* [99, 140]. The predominant mechanisms of MSC therapy are unclear. Some studies implicate homing,

engraftment and differentiation as critical for therapeutic benefit [44], and others stress the importance of secreted factors from MSCs as the key mediators [256]. To attempt to resolve this issue, we and others have tested MSC secreted factors in various disease models and compared them to whole cell transplantation. These studies have demonstrated that MSC secreted factors possess the capacity to provide therapeutic support to rodents suffering from acute diseases of inflammation (e.g. liver failure, sepsis), and can provide superior support when compared to cell transplantation [146, 171, 177]. In particular, we have seen that MSC secreted factors are responsible for a reversal of proinflammatory cytokine expression and a concomitant increase in the anti-inflammatory cytokine interleukin-10 (IL-10). To date, it has not yet been definitively resolved whether this is also the case in AKI. It has been shown in several models of AKI that these factors are capable of providing significant support [104, 105, 257], as measured by a reduction in the total injury to the renal tubule epithelium, and preservation of glomerular filtration rate and creatinine clearance in ischemic and rhabdomyolytic injury models. Tögel and colleagues demonstrated that when MSCs are transplanted in the context of AKI, very few cells engraft within hours of transplantation, and no cells can be detected in the kidneys after 24 hours [104]. Nevertheless, they still observed improvement of the structure and function of kidneys during ischemic AKI. In addition, they observed a similar reversal of the proinflammatory injurious state during AKI as we have seen in other inflammatory disease models with MSC secreted factors, as evidenced by suppression of proinflammatory cytokine expression and upregulation of the expression of anti-inflammatory mediators in the kidney at the level of mRNA, including IL-10.

In the current report, we demonstrate that MSC secreted factors are capable of protecting rats from developing severe AKI after cisplatin treatment, and can provide a survival benefit without cell transplantation. Compared to vehicle and a mock cell control, we saw a statistically significant survival benefit conferred by MSC conditioned medium (CM), together with a restoration of both histological and serum kidney function parameters. In addition, we observed a significant increase in serum IL-10 levels in animals treated with MSC-CM, and when IL-10 was neutralized using an antibody, the effects of the MSC-CM

were partially abated. The results of this study clearly indicate that in AKI, the secreted factors alone are sufficient for reversal of disease and prolonged survival.

3.2.3. Materials and Methods

3.2.3.1. Statistics

Unless otherwise noted, all experiments were repeated in quadruplicate, and all data were assessed for significance using a paired, two-tail Student's T test.

3.2.3.2. Cell Culture and Conditioned Medium

MSCs were isolated, purified, grown and characterized, and fibroblasts grown as described previously [41, 258]. All MSCs were used at passage 2-5. The conditioned medium was also collected and concentrated as described previously [42]. By convention, 1X refers to the concentration of conditioned medium achieved when 15 mL of conditioning medium was incubated in the presence of $2x10^6$ cells for 24 hours, collected, and concentrated to a final volume of 1 mL. This preparation was used for both MSCs and fibroblasts for the purposes of these studies.

3.2.3.3. Rat Model of Cisplatin-Induced Acute Kidney Injury and MSC-CM Treatment

All animal studies were performed in accordance with the animal rights policies of the Massachusetts General Hospital Subcommittee on Research Animal Care. Male SAS-SD rats (Charles River Laboratories, Wilmington, MA) weighing 275-300g were given cisplatin (7.5 mg/kg; Sigma, St. Louis, MO) dissolved in physiological saline via i.p. injection to induce AKI. At various time points after injection, MSC-CM, FB-CM, physiological saline or mixtures of MSC-CM plus anti-rat IL-10Ab (BD, Franklin Lakes, NJ; Invitrogen, Carlsbad, CA) were administered intravenously as treatments for the cisplatin-induced AKI. All MSC-CM and FB-CM doses consisted of 1 mL of 1X CM per dose, and in all cases, the concentration of IL-10Ab in each dose was 4 μg diluted in 1 mL of conditioned medium.

3.2.3.4. Tissue Histology and Staining

Rats were anesthetized with isofluorane (Hospira, Lake Forest, IL) and laparotomy was performed to access the abdominal cavity. Euthanasia was performed by severing the abdominal aorta and puncturing the diaphragm. Both kidneys were excised and rinsed with physiological saline before fixation in buffered formaldehyde (Sigma, St. Louis, MO) for one week at room temperature. The kidneys were then embedded in paraffin wax and sliced in 4 μm sections using a microtome. The sections were then analyzed by either (1) staining with hematoxylin and eosin; (2) TUNEL according to the manufacturer's instructions (R&D Systems, Minneapolis, MN); or (3) probed with PCNA antibody as described previously [259]. For the PCNA staining, we used an anti-PCNA clone PC10 (Sigma, St. Louis, MO), followed by a secondary sheep anti-mouse (1:50; Chemicon, Temecula, CA), and signal development using the Vectastain ABC kit (Vector Labs, Burlingame, CA). To determine the number of PCNA positive cells per field, representative images were taken of the kidney samples, 25 images of the medulla and 25 images of the cortex, and the numbers of positive cells per field were counted and tabulated.

3.2.3.5. Blood Urea Nitrogen (BUN) and Creatinine Measurement

All BUN and creatinine measurements were conducted with whole blood drawn from the rats and analyzed on a Renal Function Panel according to manufacturer's instructions (Abaxis, Union City, CA).

3.2.3.6. ELISAs

The ELISA kits used were provided by commercial vendors and were used according to the manufacturers' instructions (OptEIA ELISA sets; BD, Franklin Lakes, NJ).

3.2.4. Results

3.2.4.1. MSC-CM Attenuates Cisplatin-Induced AKI in Rat

In these studies, we chose to use a cisplatin model of AKI in rats. Cisplatin is used routinely in the clinic as a cancer chemotherapeutic agent, and remains the standard of care for many malignancies, including ovarian and cervical cancer, because of its strong therapeutic track record [260, 261]. However, one of the most common and dangerous complications of cisplatin administration is nephrotoxicity [262], which can lead to death associated with AKI. The primary hypothesis to be tested was whether MSC secreted factors, when used to treat lethal cisplatin administration, could provide the multifactorial support required to protect the kidneys and prevent death by organ failure.

We began our studies by optimizing the cisplatin dose required to induce 75% mortality by two weeks, and arrived upon 7.5 mg/kg i.p. [263]. We then sought to determine an effective regimen for treating AKI with MSC-CM. Previous studies illustrating the kinetics of disease onset demonstrated irreversible damage within the first 72 hours after cisplatin administration [264]. Hence, we chose five timepoints for i.v. administration of MSC-CM: 3, 9, 24, 48 and 72 hours after cisplatin was given. This regimen provided a significant survival benefit (**Figure 3.2.1**); compared to controls, MSC-CM improved survival by approximately 300% ($p < 0.05$) [41]. In addition to providing a survival benefit, MSC-CM also significantly attenuated the severity of cisplatin AKI. When animals were administered the same lethal dose of cisplatin, BUN and creatinine rose dramatically in vehicle treated rats by day 3, and continued to climb by day five, indicative of the severity of disease and irreversibility of the injury (**Figure 3.2.2a and 3.2.2b**). However, when the animals were treated with MSC-CM as described above, neither BUN nor creatinine rose dramatically at any point during the first five days after cisplatin administration. This would indicate that MSC-CM prevented extensive injury of the kidneys and preserved enough of the architecture and function of the kidneys such that clearance of BUN and creatinine was not irreversibly affected.

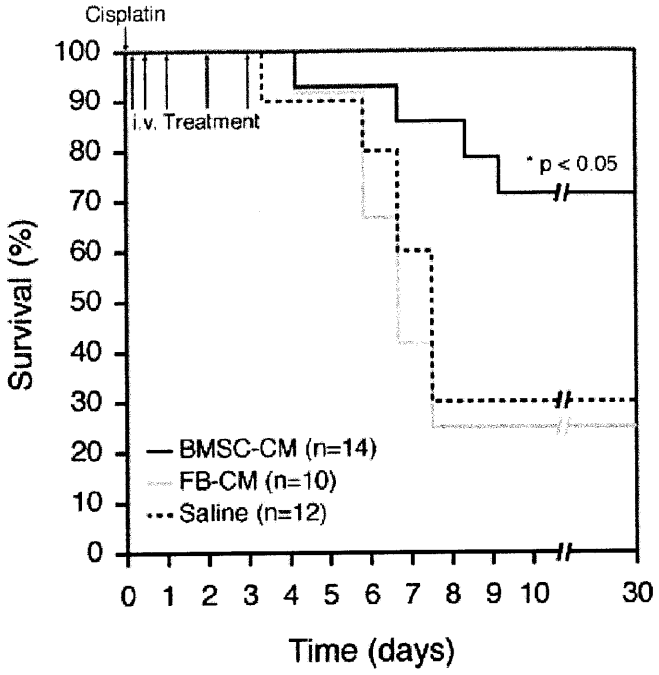


Figure 3.2.1 – MSC-CM confers a significant survival benefit to rats undergoing lethal cisplatin AKI. At hour 0, cisplatin dissolved in physiological saline (0.75 mg/mL) was administered i.p. to male, SAS-SD rats at a lethal dose (7.5 mg/kg). At hours 3, 9, 24, 48 and 72, 1 mL of either physiological saline (n=12), fibroblast conditioned medium (n=10) or MSC conditioned medium (n=14) were administered i.v. to the cisplatin-induced AKI rats. They were monitored for surivival for 30 days. *p < 0.05 for MSC-CM compared to both FB-CM and saline by Logrank test.

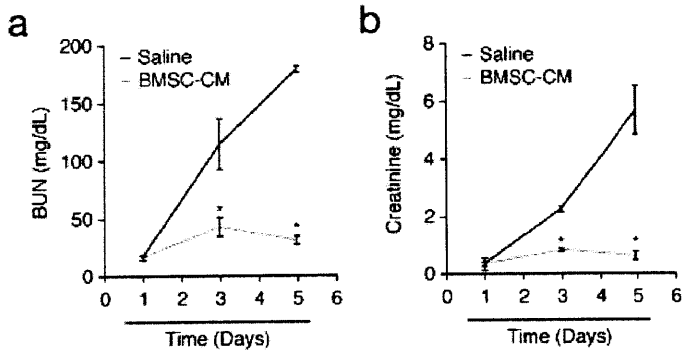


Figure 3.2.2 – MSC-CM prevents onset of severe AKI. Blood was collected from rats subjected to lethal cisplatin (7.5 mg/kg) i.p. via tail snip 1, 3 and 5 days following cisplatin administration. The rats were treated at 3, 9, 24, 48 and 72 hours following cisplatin administration, with 1 mL of either physiological saline (n=4), or MSC conditioned medium (n=3) i.v. (a) BUN and (b) creatinine were measured to monitor the extent of renal injury as a function of time after cisplatin. *p < 0.01 by Student's T-test.

3.2.4.2. MSC-CM Protects the Kidney from Cisplatin-Induced Nephrotoxicity

From the results of the renal function markers, BUN and creatinine, it seemed likely that the MSC-CM treatment was protecting the architecture of the kidney, thereby preserving a normal clearance rate. When the rats were subjected to a lethal dose of cisplatin and then

treated with saline, hematoxylin and eosin staining of the kidneys revealed cortical damage in the form of collapsed Bowman's capsule and proximal tubular necrosis, and medullary damage in the form of extensive tubular necrosis, deposition of debris and intermediate tubule casts, all consistent with cisplatin-mediated AKI (**Figure 3.2.3**). This destruction of the tubule architecture of the kidney, Bowman's capsule collapse, and obstruction of the loops of Henle could explain why the BUN and creatinine rose so dramatically by day 5. The injury was so severe that upon excision, the kidneys were grossly hemorrhagic and the normal tissue firmness was replaced with a soft and shapeless character. The kidneys of the rats administered MSC-CM, on the other hand, sustained significantly less injury. H&E analysis revealed only minor damage in the form of moderate tubular necrosis, loss of brush borders and denuding of tubular epithelium, but minimally apparent casts or accumulated debris. On the whole, the kidney was able to retain an non-obstructed structure, clearly advantageous to maintaining clearance and preserving normal kidney function even despite toxic injury.

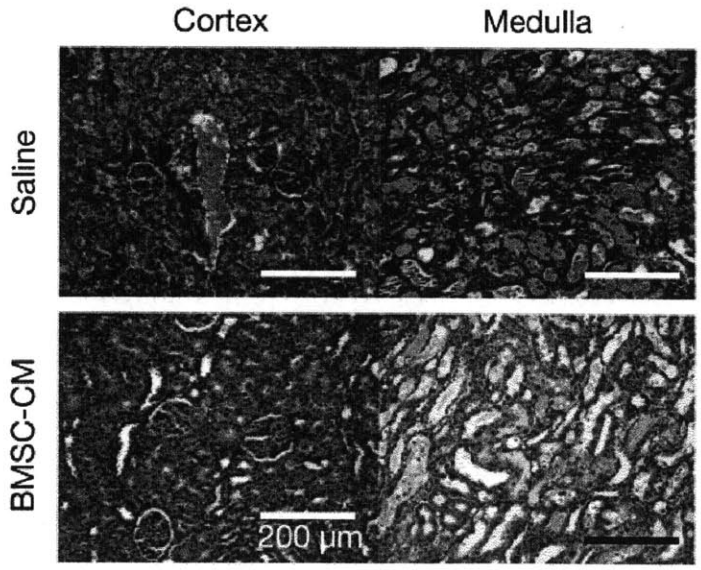


Figure 3.2.3 – MSC-CM protects the native architecture of the kidney during AKI. Hematoxylin and eosin staining of sections of kidneys from rats administered a lethal dose of cisplatin (7.5 mg/kg) and sacrificed at 5 days following cisplatin administration. The rats were treated at 3, 9, 24, 48 and 72 hours following cisplatin administration, with 1 mL of either physiological saline (n=4), or MSC conditioned medium (n=3) i.v.

We next sought to analyze the type of injury sustained by the kidneys during cisplatin toxicity. We hypothesized that in addition to frank necrosis induced by cisplatin toxicity, apoptosis was also present in the kidneys, even as late as 5 days after the cisplatin injection. We performed TUNEL on these day-5 kidney sections and noted extensive

apoptotic foci in the medulla of the vehicle treated kidneys (**Figure 3.2.4**). Periodic apoptotic cells were observed in the MSC-CM treated kidneys, but 10X fewer than found in the vehicle treated kidneys, indicating far fewer apoptotic events. This would suggest that one mechanism by which the MSC-CM protects the architecture of the kidney is by preventing apoptosis.

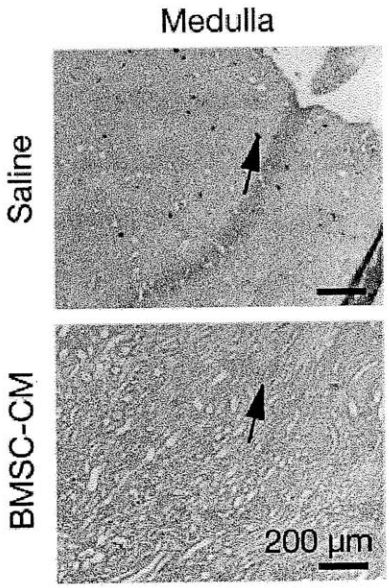


Figure 3.2.4 – MSC-CM prevents apoptosis in the loop of Henle. TUNEL staining was performed on sections of formalin fixed and paraffin-embedded kidneys from rats administered a lethal dose of cisplatin (7.5 mg/kg) and sacrificed at 5 days following cisplatin administration. The rats were treated at 3, 9, 24, 48 and 72 hours following cisplatin administration, with 1 mL of either physiological saline (n=4), or MSC conditioned medium (n=3) i.v. Arrows indicate positively stained nuclei.

We then looked into the prevalence of regeneration in the kidneys to test the hypothesis that MSC-CM enhances regeneration in the tissue, which contributes to reversal of the disease and recovery of the animals. We probed for PCNA and found that indeed, regeneration was far more extensive in the MSC-CM treated kidneys than healthy control kidneys that only showed scarce dividing cells (less than one per 10X field on average) (**Figure 3.2.5**). Interestingly, PCNA analysis revealed that vehicle-treated kidneys were also undergoing extensive regeneration. We quantified the number of PCNA positive cells in each 20X field, and found that the extent of regeneration in vehicle-treated kidneys was significantly higher than in MSC-CM treated kidneys. As will be discussed below, we hypothesize this may be a function of the total damage incurred by the kidney, as extensive damage will require more dividing cells to replenish lost cells compared to moderate or minimal damage.

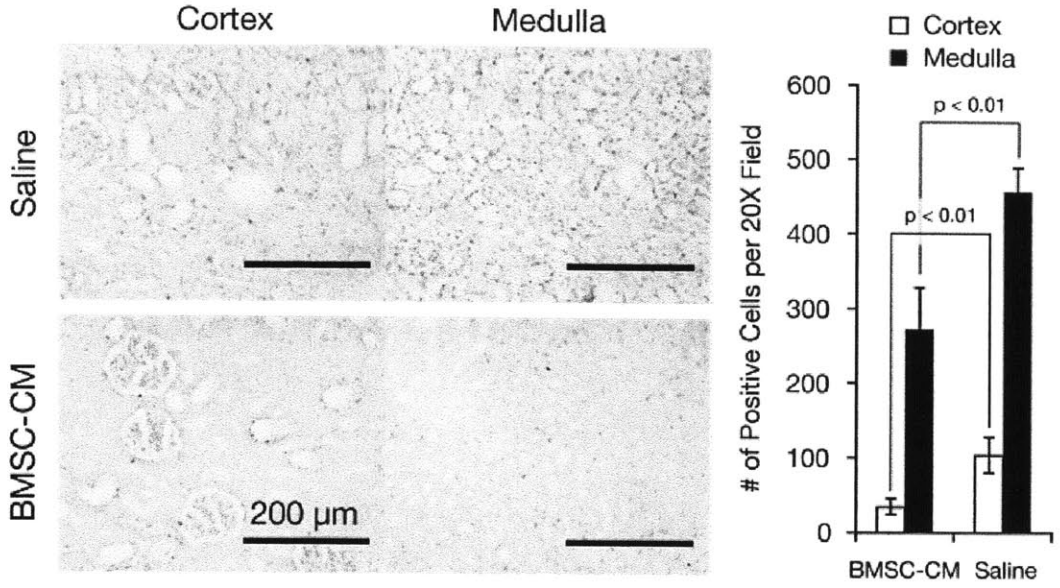


Figure 3.2.5 – Vehicle treated animals require more regeneration to overcome injury to the kidneys during AKI. PCNA staining was performed on sections of formalin fixed and paraffin-embedded kidneys from rats administered a lethal dose of cisplatin (7.5 mg/kg) and sacrificed at 5 days following cisplatin administration. The rats were treated at 3, 9, 24, 48 and 72 hours following cisplatin administration, with 1 mL of either physiological saline (n=4), or MSC conditioned medium (n=3) i.v.

3.2.4.3. IL-10 is Important for MSC-CM Therapy in AKI

Since we saw such a strong response in the rats to MSC-CM in AKI, we next wanted to begin to explore mechanistic implications of MSC-CM therapy, in particular how MSC-CM might influence the immune state of the animal during AKI. Our previous work revealed that during liver failure, the inflammatory state of rats treated with MSC-CM was reversed compared to vehicle controls [42]: the vehicle treated rats developed high levels of IL-1β, TNF-α, IL-6 and IL-1ra, and exhibited low levels of IL-10, while MSC-CM treated rats developed high levels of IL-10 and lower levels of IL-1β, TNF-α, IL-6 and IL-1ra. In other models as well, IL-10 upregulation has been shown to be a hallmark of MSC therapy [146], and has been shown to influence the expression of IL-1β, TNF-α and IL-6 *in vitro* and *in vivo* [104, 265]. In AKI, we therefore hypothesized that MSC-CM was causing enhanced IL-10 expression in a similar manner. The serum level of IL-10 in MSC-CM treated rats three days after cisplatin administration was significantly higher than that in vehicle controls

(**Figure 3.2.6a**). To confirm that the IL-10 we measured did not arise from the MSC-CM that we injected, we measured via ELISA the amount of IL-10 in MSC-CM. Even though the MSC-CM was from human cells and the IL-10 we measured in the animals was rat, we nevertheless wanted to confirm there was no cross-reactivity in the ELISA and measured the MSC-CM independently. We found that the minute positive reactivity of MSC-CM in the ELISA could not account for the IL-10 seen in the serum (**Figure 3.2.6b**).

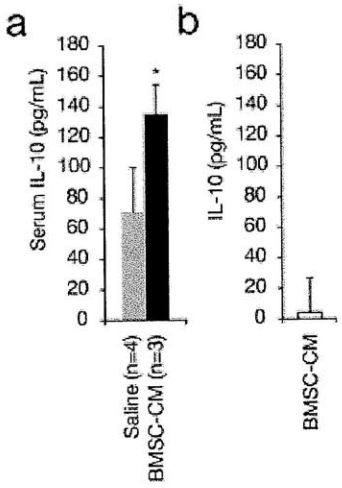


Figure 3.2.6 – MSC-CM significantly upregulates IL-10 in AKI rats, and MSC-CM is not an exogenous source of IL-10. (a) Rat serum IL-10 was measured via ELISA from rats administered a lethal dose of cisplatin (7.5 mg/kg) and then treated with either vehicle or MSC-CM at 3, 9, 24, 48 and 72 hours after cisplatin administration. Blood was collected via tail snip, and the serum was analyzed on day 3. *$p < 0.05$ by Student's T-test. (b) IL-10 was measured via ELISA in whole MSC-CM.

Finally, to test the extent to which IL-10 may be responsible for the therapeutic effectiveness of the MSC-CM treatment in AKI, we tested the same survival model of cisplatin AKI (7.5 mg/kg) with MSC-CM coadministered with neutralizing IL-10 antibody at 4 μg per dose. **Figure 3.2.7** shows the diminished effect of MSC-CM on the survival of animals when co-administered with neutralizing IL-10 antibody. Compared with the survival associated with MSC-CM alone, 30% fewer animals survived when IL-10 antibody was present. However, it should be noted that when tested for significance, the p-values of the differences between MSC-CM vs. MSC-CM with IL-10 antibody were greater than 0.05, suggesting trends rather than significant increases or decreases in survival. Nonetheless, together these results support the hypothesis that IL-10 is an important mediator of MSC-CM therapy, and suggest that MSC-CM can stimulate endogenous anti-inflammatory processes to aid in the reversal of AKI.

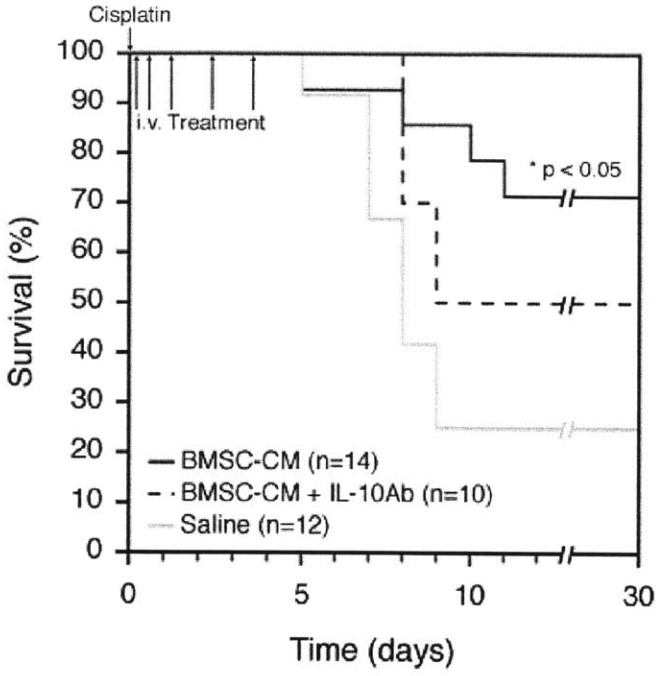


Figure 3.2.7 – IL-10 antibody abates the survival benefit of MSC-CM administered to rats undergoing lethal cisplatin AKI. At hour 0, cisplatin dissolved in physiological saline (0.75 mg/mL) was administered i.p. to male, SAS-SD rats at a lethal dose (7.5 mg/kg). At hours 3, 9, 24, 48 and 72, 1 mL of either physiological saline (n=12), MSC conditioned medium (n=14) or MSC conditioned medium containing 4 μg/mL neutralizing anti-rat IL-10 antibody (n=10) were administered i.v. to the cisplatin AKI rats. They were monitored for surivival for 30 days. *$p < 0.05$ for MSC-CM compared to saline by Logrank test.

3.2.5. Discussion

We have shown that MSC-CM alone can provide substantial support in AKI. Several studies have shown that transplanted MSCs can provide survival benefit in AKI akin to what we observed. In one study, i.v. administration of human MSCs ($2x10^7$ cells/kg) in NOD/SCID mice at 24 hours following lethal cisplatin administration enhanced survival from 0% to 40% [112]. In another, i.p. administration ($2x10^8$ cells/kg) was demonstrated to be sufficient for providing survival benefit to NOD/SCID mice, enhancing survival from 10% to 47% [266]. In our study, we used the secreted factors from the equivalent of $3x10^7$ cells/kg, which is within the same order-of-magnitude as other work demonstrating i.v. administration of MSCs, which is why we might expect a similar result in terms of protection during AKI. Nevertheless, this is not a direct comparison as the MSC-CM consists of secreted factors from only 24 hours of conditioning, compared to permanent engraftment of transplanted MSCs and continuous secretion of factors over time from the engrafted cells [267].

Our results are consistent with other studies of MSC-CM in AKI, and show for the first time that human MSC-CM can provide a survival benefit in the context of AKI. Bi and colleagues showed that in addition to MSC transplantation conferring a survival benefit to mice undergoing cisplatin AKI, mouse MSC-CM also provided a 60% increase in survival [268]. We observed a 300% improvement in survival, which we hypothesize could be attributed to species differences of the CM [44]. However, since the study of Bi et al. was executed differently than ours in terms of AKI rodent species, mode of administration of MSC-CM, treatment regimen and MSC-CM harvesting method, it is difficult to compare the results of the studies directly. Also, significant reversal of AKI has been reported that resulted from administration of the ultracentrifuged fraction of MSC-CM in mice undergoing rhabdomyolytic AKI [257]. The authors present evidence that microvesicles in the ultracentrifuged fraction provide benefit by horizontal gene transfer that is diminished with the co-administration of RNAse, as evidenced by histology, BUN/creatinine and electron microscopy. Nevertheless, the authors did not explore whether the ultracentrifuged fraction provides a survival benefit, nor do they report whether the whole conditioned medium can provide a similar or superior benefit. In all, our results here have demonstrated the best improvement of survival thus far of any MSC study to our knowledge, and show that the secreted factors alone are sufficient for AKI resolution.

Our previous work has shown that the secreted factors from MSCs, either administered in the form of conditioned medium (CM) or delivered continuously from an extracorporeal bioreactor, are capable of providing survival benefit to animals undergoing fulminant hepatic failure [41, 42]. We observed reversal of the pro-inflammatory cytokine profile in these animals, with a marked increase in the anti-inflammatory cytokine IL-10. IL-10 has been implicated in MSC therapy previously in sepsis [146], and has been cited as one of the more influential factors upregulated during MSC-mediated T-cell suppression [99, 269]. In sepsis, MSCs were shown to upregulate IL-10 in macrophages via prostaglandin E2-mediated reprogramming. Others groups have shown that certain factors secreted by MSCs can directly lead to IL-10 production [256], including TGF-β [270] and IL-6 [271], two proteins that are highly expressed by MSCs [172, 272]. IL-10 has also been shown to be

effective as a therapeutic when exogenously delivered to rodents undergoing AKI [273], confirming the important role that IL-10 can play in reversing the disease. It comes as no surprise, therefore, that we observed upregulation of IL-10, and blocking the cytokine caused a decrease in the survival of animals treated with MSC-CM.

In an effort to leverage what we have learned about MSC-CM and begin to translate it to clinical use, we have recently been working to scale up the extracorporeal bioreactor we developed to treat fulminant hepatic failure in rodents [41] to accommodate human use during dialysis for AKI [274]. In our studies of hepatic failure, we discovered that continuous delivery of MSC secreted factors from a bioreactor can provide a superior therapeutic benefit compared to bolus administration of MSC-CM [41]. Hence, we developed a platform that will integrate with existing dialysis circuits to allow for delivery of MSC secreted factors directly into the bloodstream of patients with AKI undergoing dialysis. When seeded in the bioreactor, we showed the MSCs are capable of remaining viable and functional while retaining their undifferentiated phenotype under flow conditions mimetic of clinical operation [274], which suggests that scale-up of the prototype device from our liver failure studies will likely result in a similarly active device for clinical use. The results we report here further support the hypothesis that the secreted factors from MSCs delivered into the bloodstream of animals suffering from AKI can fundamentally change the outcome of those suffering from the disease, and provide reasonable evidence that the MSC bioreactor platform could be effective in treating human AKI.

One unexpected result was the significantly enhanced regeneration in vehicle-treated kidneys compared to MSC-CM-treated. This is contrary to earlier findings that MSCs promote regeneration in kidneys during AKI [112], as clearly evidenced by PCNA staining. This is also in contrast to studies we have previously performed that suggested MSC-CM promotes regeneration in the context of fulminant hepatic failure in rat [42]. In these latter studies, there was clearly enhanced regeneration in the livers of rats treated with MSC-CM compared to vehicle controls. In both cases, however, the time at which the animals are sacrificed and analyzed is important to consider. In our liver failure studies, the rats were

sacrificed and tissue extracted for analysis only 48 hours after the final dose of D-galactosamine, the agent used to induce FHF, and in the AKI study, the mice were sacrificed four days after cisplatin. In the current study, the animals were sacrificed 48 hours after the final dose of MSC-CM, which is five days after the induction of AKI with cisplatin. Therefore, it is possible that had the animals been sacrificed earlier in these studies, we might also observe a higher rate of regeneration. Tögel et al. observed that PCNA staining is significantly higher in kidneys from rats treated with MSCs 24 hours after reflow during ischemia-reperfusion AKI [104]. It is also possible that the higher rate observed in the vehicle-treated kidneys is a consequence of the prevalent damage to those kidneys that is not suffered to the same extent in those treated with MSC-CM. Finally, since PCNA staining may not be specific to proliferating cells per se and could also be indicative of DNA repair, which would be required extensively in the context of cisplatin AKI, it is possible that discrepancies in PCNA staining between the MSC-CM treated and saline treated animals could be suggestive of differences of the two therapeutic approaches in terms of DNA damage. In any case, it is clear from the physiological response of the animals to MSC-CM that the regeneration and/or repair that is taking place by day 5 is sufficient to restore function to their kidneys to normal levels.

In conclusion, we have demonstrated that MSC secreted factors, independent of cell transplantation, are capable of stimulating endogenous anti-inflammatory programs via IL-10, leading to a significant survival benefit to rats undergoing cisplatin AKI. These results suggest that the primary mechanism of MSC therapy is based on the secreted factors of the cells, which should aid in the optimization of proper therapeutic protocols for treatment of AKI patients with MSCs. It also suggests that alternative approaches to transplantation might also prove successful for administering MSC therapy, including administration of the conditioned medium as a direct injectable, or continuous delivery of these secreted factors from bioreactors into the bloodstream of AKI patients, thereby circumventing potential risks associated with transplantation [151, 152]. In all, these results present a promising and innovative new approach to treating this deadly disease that leverages the natural strengths of the MSC secretome.

3.3. Phenotypic and Functional Characterization of Human Bone Marrow Mesenchymal Stem Cells in Hollow Fiber Bioreactors

3.3.1. Abstract

The transplantation of human mesenchymal stem cells (MSCs) is a novel immunotherapeutic approach that is currently being explored in many clinical settings. Evidence suggests that the efficacy of cell transplantation is directly associated with soluble factors released by human MSCs. In order to harness these secreted factors, we integrated MSCs into large-scale hollow-fiber bioreactor devices in which the cells (separated by a semipermeable polyethersulfone (PES) membrane) can directly and continuously release therapeutic factors into the blood stream. MSCs were found to be rapidly adherent and exhibited long-term viability on PES fibers. The cells also preserved their immunophenotype under physiologic fluid flow rates in the bioreactor, and exhibited no signs of differentiation during device operation, but still retained the capacity to differentiate into osteoblastic lineages. MSC devices released growth factors and cytokines at comparable levels on a per cell basis to conventional cell culture platforms. Finally, we utilized a potency assay to demonstrate the therapeutic potential of the collected secreted factors from the MSC devices. In summary, we have shown that culturing MSCs in a large-scale hollow fiber bioreactor is feasible without deleterious effects on phenotype, thus providing a platform for collecting and delivering the paracrine secretions of these cells.

3.3.2. Introduction

Mesenchymal stem cells (MSCs) are a subpopulation of adherent bone marrow cells that have a long-standing period of clinical testing in a number of disease states ranging from osteogenesis imperfecta to graft-versus-host disease [9, 35, 36, 128, 275]. Their therapeutic use has expanded in recent years after it was observed that MSC transplants

could modulate the immune response to tissue injury [119, 120]. The primary method of administration has been the intravenous transplantation of these cells. However, the viability of MSCs after intravenous transplantation exponentially declines [14, 15] limiting the therapeutic window of treatment. Also, the possibility of unwarranted side-effects of transplants, such as maldifferentiation or systemic reduction of immunosurveillance [151, 152, 158, 159], remains to be determined in ongoing longitudinal patient trials.

A corresponding line of research has emerged to define the mechanism of action of MSC transplants. Many studies have demonstrated that secreted factors released by MSCs during transplantation alter the function of neighboring cells, which can result in a tissue-sparing, anti-inflammatory outcome (reviewed in [267]). These secreted factors can be broadly classified by whether they are basally produced, inducible, or the products of metabolic conversion of local substrates. For example, MSCs naturally secrete IL-1ra and VEGF [276, 277], can be induced by toll-like receptor activation to secrete prostaglandin E2 and sTNFR1 [146, 278], or can catabolize tryptophan into a T cell suppressant, kynurenine, by the action of indoleamine 2,3-dioxygenase [176, 279]. These reports solidify the concept of MSCs being a dynamic reservoir of soluble factors that require suitable methods to deliver this molecular therapy in a controlled manner.

Some investigators have evaluated the use of MSC secretions by administering MSC conditioned medium (MSC-CM) by various routes. Initial work demonstrated that local injections of MSC-CM could supplant the effect of a MSC transplant in protecting cardiac muscle viability and function in ischemic hearts during acute phases of recovery [280, 281]. Our group demonstrated that systemic delivery of MSC-CM by means of an intravenous bolus led to an improvement in short-term survival of rats undergoing organ failure [41]. We also designed prototype flat-plate bioreactors to create a single platform for MSCs to secrete factors continuously into the bloodstream. These extracorporeal support devices showed improved survival in therapeutic trials in rodents [41, 177]. Collectively, these studies indicate that MSCs can be integrated into blood-contacting devices and can elicit therapeutic effects by paracrine action alone.

On this basis, we evaluated human scale hollow fiber bioreactors as a means to harvest and/or deliver MSC secreted factors in a clinically-relevant continuous blood flow device. These devices differ from our lab-scale prototype in that MSCs are seeded onto 3D hollow fibers and are separated from continuous fluid flow by a porous hollow fiber membrane. In this report, we have demonstrated that MSCs retain their phenotype in hollow fiber bioreactors and their secretions maintain bioactivity during their culture time in devices.

3.3.3. Materials and Methods

3.3.3.1. Mesenchymal Stem Cell Isolation

Primary human MSCs were derived from whole human bone marrow aspirates (Lonza, Basel, Switzerland). Bone marrow was firstly diluted in a 1:1 ratio with sterile phosphate buffered saline (PBS). The diluted marrow was subsequently added on top of an equivalent volume of Ficoll-Paque™ PREMIUM (GE Healthcare, Uppsala, Sweden); this was then spun at 1500 RPM for 30 minutes. Centrifugation separated the mixture into four density partitions: serum/PBS, buffy coat, Ficoll, and hematocrit. The top layer of serum/PBS was first carefully aspirated as to not disrupt the buffy coat layer. Once enough volume was removed, the buffy coat was transferred to another tube. An equal amount of medium (recipe in 2.2. Cell culture) was added and the mixture spun at 1500 RPM for 5 minutes. The supernatant was removed and replaced with 25 mLs of culture medium and the total number of cells was determined using hemocytometer. Cells were then diluted to a plating density of approximately 10,000 cells/cm^2 in T175 (Corning) flasks in a 37°C, 10% CO_2 incubator. Adherent cells were allowed to grow for one week before the medium was changed and non-adherent cells removed. Cells were then cultured for another week after which the medium was changed every three days. Once at a confluency of 70-80%, cells were frozen down in culture media containing 10% DMSO (v/v) and individual lots from this master cell bank were used for experimentation.

3.3.3.2. Cell culture

MSCs were used between passages 1-5 and grown in medium composed of: alpha-modified Minimum Essential Medium Eagle (Sigma, St. Louis, MO), 20 mg/L gentamycin (Sigma), 15% FBS (Hyclone, Logan, Utah), 1 ug/L rhFGF-basic (R&D Systems, Minneapolis, MN), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) and titrated to a pH of 7.4. Cells were grown at 37°C in T175 culture flasks in a 10% CO_2, humidified incubator. Medium was replaced every three days and cells were replated at a split ratio between 1:2 and 1:10 using 0.25% trypsin-EDTA after they reached 80-90% confluency.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole human blood in the same manner as MSCs, described above, using a Ficoll-Paque™ separation technique. PBMC culture medium was composed of: RPMI 1640 (Sigma), 10% heat inactivated FBS (Hyclone), 50 U/ml penicillin (Sigma), and 50 μg/ml streptomycin (Sigma) and titrated to pH 7.4. Cells were grown at 37°C in a 5% CO_2, humidified incubator.

3.3.3.3. Hollow fiber bioreactor seeding and operation

Hollow fiber dialyzers were generously donated by NxStage (Lawrence, MA). The encased ultrafiltrate hollow fibers were constructed from polyethersulfone (PES) and spanned a total surface area of 1.6 m^2. Each fiber had a lumen diameter of 200 μm. For initial cell seeding experiments, the bioreactor was disassembled and individual hollow fibers were sterilely organized into a bundle of approximately 25 fibers and placed in 6 cm tissue culture dishes for incubation with a cell suspension. Approximately 3.0 x 10^5 cells in a volume of 200 μL of culture medium were applied onto each 1x1 cm fiber bundle and allotted a period of three hours for attachment in an incubator. After which, 6 mLs of medium was added and the cells were allowed to grow on the fibers for the duration of the experiment. This method of seeding ensured that the vast majority of the cells were attaching to the fibers and not the

plate since the cell suspension volume was just sufficient enough to wet the fibers and not seep onto the plate.

In preparation for cell seeding in the assembled device, the intra/extracapillary spaces were manually flushed with 300 mLs of sterile PBS. Excess fluid was removed by aspiration. The intracapillary space was then filled with culture medium and was considered full when medium was seen exiting the other port on the device. Cells were taken from frozen stocks, thawed, centrifuged, and resuspended in a volume of approximately 50 mLs of fresh culture medium and counted. The cell suspension was syringe injected into the extracapillary space through one dialysate port. Extra medium was added until the entire extracapillary space was filled as assessed by medium evacuation from the opposite port. The device was then placed in an incubator overnight to allow for the adherence of cells to the fibers. The following day, the extracapillary space was manually flushed with 200 mLs of PBS to remove any non-adherent or dead cells and the volume again replaced with culture medium. The device was then attached to the closed loop perfusion system consisting of a medium reservoir and peristaltic pump (pump drive – Masterflex L/S; pump head – Masterflex easy-load 3; Cole-Parmer Instrument Co., Vernon Hills, IL) and then placed back in the incubator, and allowed to run for the duration of the experiment. Intracapillary flow rate was set to 200 mL/min with a resulting extracapillary flow rate of 10 mL/min.

3.3.3.4. Fluorescent and Scanning Electron Microscopy

To assess the ability of cells to grow on hollow fibers in a static environment, live cell imaging was performed as per the vendor provided protocol for the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen/Molecular Probes, Carlsbad, CA). Approximately, $3x10^5$ cells were allowed to adhere to fiber samples overnight in standard culture medium prior to staining and fluorescent imaging. The morphology of PES hollow fiber and adhered MSCs were characterized under scanning electron microscope (Ultra55, Zeiss). MSCs growing on the fibers were fixed with 2.5% glutaraldehyde and then serially dehydrated in increasing

concentrations of ethanol solution; 20, 50, 70, 90, 95 and 100%. Before imaging, the samples were sputter-coated with Au target for 2 min in 20mA (HAR-024, Cressington).

3.3.3.5. Osteoinduction analysis

MSCs were trypsin-extracted from the device after a 48-hour perfusion and analyzed for signs of osteogenic differentiation. The entire bioreactor was cleared of media, flushed with PBS, and filled with trypsin. Cells were allowed to detach for half an hour with mechanical agitation applied every 10 minutes. Cells were removed through syringe aspiration. This aspirate was then added to an equal volume of media, centrifuged, and the number of cells counted. To test for signs of differentiation, $2x10^5$ cells were firstly plated onto a 2.5 x 7.5 cm glass slide and allowed to adhere overnight. The following day the cells were stained with Alizarin Red S (Sigma) as per standard protocol and imaged. In a separate set of experiments we tested their ability to differentiate after device operation. MSCs ($5x10^4$) cells were plated in a Lab-Tek Chamber Slide (Nunc, Rochester, NY) cell culture system and grown in StemPro Osteocyte/Chondrocyte differentiation medium (Gibco/Invitrogen) for a period of approximately 3 weeks with complete medium changes every 3 days. The cells were visually assessed for morphological changes, stained with Alizarin Red S, and imaged once again to verify differentiation.

3.3.3.6. Metabolite analysis

Analysis of two secreted metabolites was used as additional validation of cell viability in the hollow fiber dialyzer. Measurements of glucose (Stanbio, Boerne, TX) and lactate (Trinity Biotech, Bray, Ireland) were performed per vendor protocols. Medium samples were taken over a period of 48 hours from a bioreactor seeded with 200 million cells and perfused with an extracapillary flow rate of 10 mL/min; samples were stored at 4°C prior to analysis.

3.3.3.7. Growth factor and cytokine analysis

To further characterize the seeded MSCs in the hollow fiber dialyzer, measurements of interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) were used. Medium samples collected at 24 and 48-hour time points from a bioreactor seeded with 200 x 10^6 cells and perfused with extracapillary flow rate of 10 mL/min were stored at 4°C prior to ELISA analysis as per BD OptEIA™ protocol (BD Biosciences, San Jose, CA).

3.3.3.8. Flow cytometry

Following a 48-hour perfusion, cells were removed from the bioreactor using 0.25% Trypsin-EDTA and analyzed for phenotypical markers to validate preservation of a MSC phenotype. Cells were stained with BD Pharmigen™ CD44, CD29, CD73, and CD11b antibodies (BD Biosciences) after which flow cytometry was performed using standard operating procedures (Cell Lab Quanta™ SC, Beckman Coulter, Brea, CA).

3.3.3.9. Potency assay

Approval for the collection of blood from healthy volunteers was obtained from the Institutional Review Board of Massachusetts General Hospital. A potency assay ([258]; see Appendix) was performed in order to validate the influence of MSC secreted factors on peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were isolated by Ficoll-Paque™ separation from whole human blood. Cells were plated in round bottom 96-well plates at 1x10^5 cells/well cells in 50 μL of complete RPMI medium. 50 μL of MSC growth medium or MSC-CM from a cell-seeded bioreactor was then added to each well bringing the volume up to 100 μL. Cells were incubated for 18 hours, after which 50 μL of 30 μg/mL lipopolysacharide (LPS) (10 μg/mL final concentration) was added and incubated for 5 hours. The plate was then spun down at 1500 RPM and the supernatant removed and

stored at -80°C prior to analysis for IL-10 release. IL-10 levels were measured by standard ELISA methods (BD Biosciences).

3.3.4. Results

3.3.4.1. MSCs adhere and retain fibroblastoid morphology on polyethersulfone hollow fibers

The predominant fiber material used in clinical bioreactors is polyethersulfone (PES). PES is a standard material used in bioreactors and filtration membranes for numerous reasons including high thermal and chemical resistances, exceptional biocompatibility, and low protein binding. We first assessed if human MSCs could adhere to this material without the use of a cell-permissive coating. Cells were initially seeded in a static environment on fibers isolated from a hollow-fiber bioreactor. The efficiency of static seeding was approximately 80%. This was determined through viable cell counting and the subtraction of cells found in suspension from the initial number of cells seeded on a fiber bundle after 24 hours of incubation (data not shown). **Figure 3.3.1a** illustrates the initial adherence of viable human MSCs after 24 hours visualized by calcein AM staining. Throughout the five-day time course, viable cells began to spread along the circumference of the fiber bundles (**Figure 3.3.1b and 3.3.1c**). Scanning electron microscopy imaging validated preserved fibroblastoid morphology with a classical spindle-like shape (**Figure 3.3.1d**) and transversal cell attachment (**Figure 3.3.1e**). MSCs were also found to attach to other similar substrates such as polysulfone and cellulose that are less often used in bioreactors (data not shown).

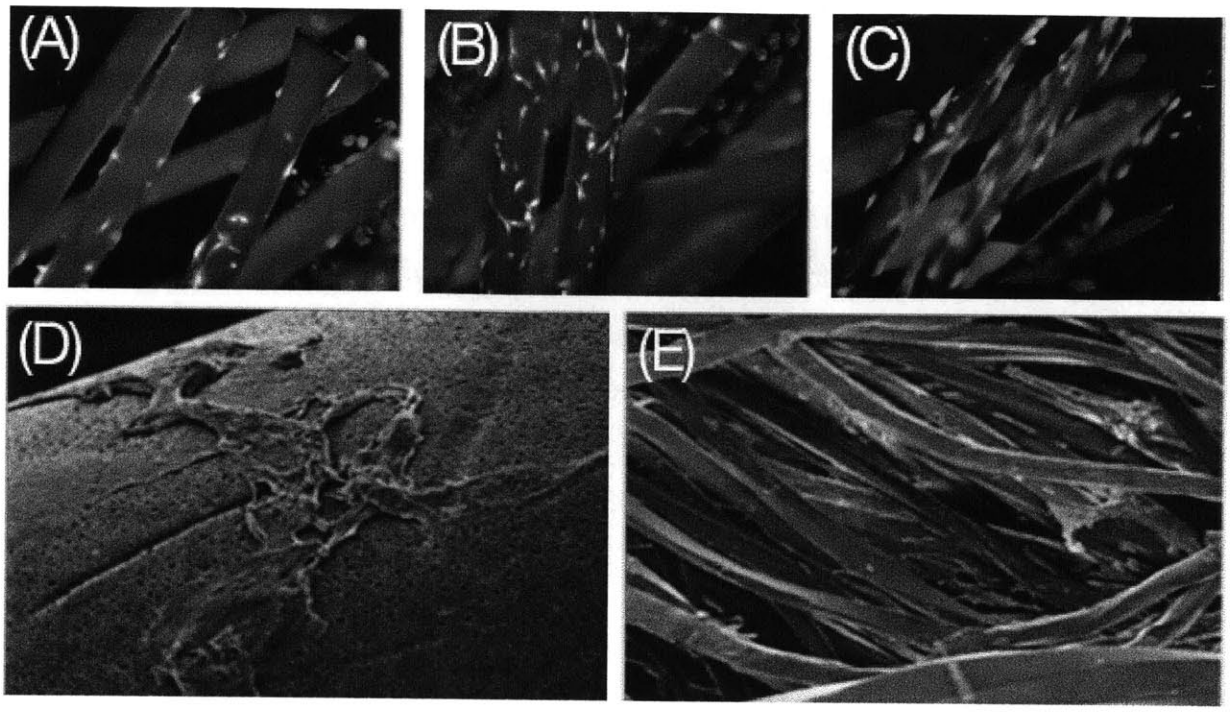


Figure 3.3.1 – MSC adhere to polyethersulfone hollow fibers and remain viable after long-term culture. Fluorescent micrographs of MSC adherence and morphology on PES hollow fibers after (A) 24 hours, (B) 48 hours, and (C) 6 days. Cells were stained with Calcein AM for viability. Scanning electron microscopic images show (D) MSCs with typical fibroblastoid morphology within the constructs of a (E) complex three-dimensional environment.

3.3.4.2. MSCs are viable in a hollow fiber bioreactor under flow conditions

There are two distinct compartments within a human-scale hollow fiber bioreactor that contain different chemical environments. Blood is typically flowed at a velocity of 100-400 mL/min through the lumen of the hollow fibers in the device. An ultrafiltrate is driven through the fiber pores into the extraluminal space in a pressure-dependent manner. The membrane pores have a molecular weight cut-off of less than 50 kDa and thereby deflect large macromolecules. We inoculated MSCs into the extraluminal compartment of the hollow-fiber bioreactor and evaluated their viability under clinical perfusion conditions. Approximately 200 x 10^6 human MSCs were seeded by gravity flow into the extraluminal space and allowed to adhere on the fiber bundles overnight before beginning perfusion. After cell adhesion to the fibers, these devices were integrated into a fluidic circuit that

enabled serial testing of the device effluents over time (**Figure 3.3.2a**). To increase the throughput of experimentation, we also constructed parallel reactor modules with minimal hardware components (**Figure 3.3.2b**). A clinically-relevant intraluminal flow rate of 200 mL/min perfused the device for 48 hours with an extraluminal flow rate of 10 mL/min. Medium was sampled daily from the reservoir and the viability of human MSCs was assessed through metabolic utilization of medium substrates. Glucose levels decreased over the 48-hour time course while lactate rose over time. These data quantifying glucose consumption and concomitant lactate production indicate viable cells that are employing glycolytic metabolism within the bioreactor (**Figure 3.3.2d**).

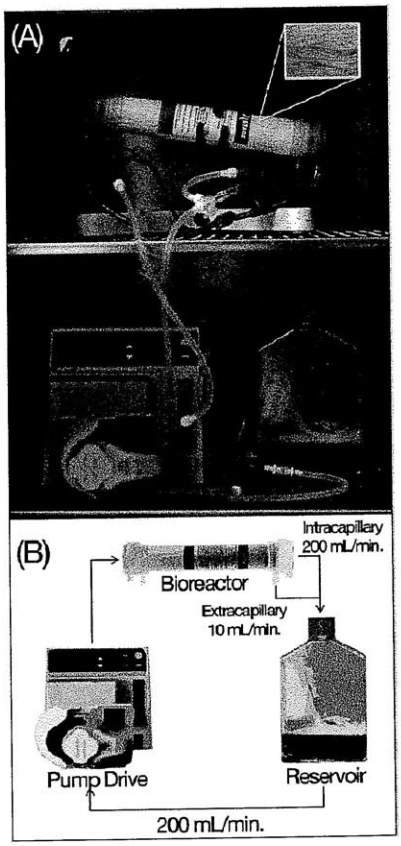


Figure 3.3.2 – MSCs are metabolically active during device operation. (A) Photograph of multi-device operation *in vitro*. (B) Schematic of a recycling flow circuit with an intracapillary flow rate of 200 mL/min. and extracapillary flow rate of 10 mL/min. MSCs are seeded on the extraluminal space.

3.3.4.3. MSCs express an undifferentiated phenotype after exposure to bioreactor culture

Once we determined that MSCs were indeed viable over time in bioreactor culture, we next evaluated if their phenotype had changed. We isolated cells by trypsin after two days of bioreactor culture at the same rates described previously and assessed the immunophenotype and osteogenic differentiation of human MSCs after device operation (**Figure 3.3.3a**). Cells stained positive for CD44 and CD73, but negative for CD11b and CD45 (**Figure 3.3.3b**) consistent with an established surface phenotype of human MSCs [45]. These results suggest human MSCs retain their immunophenotype under flow in a three-dimensional hollow fiber configuration.

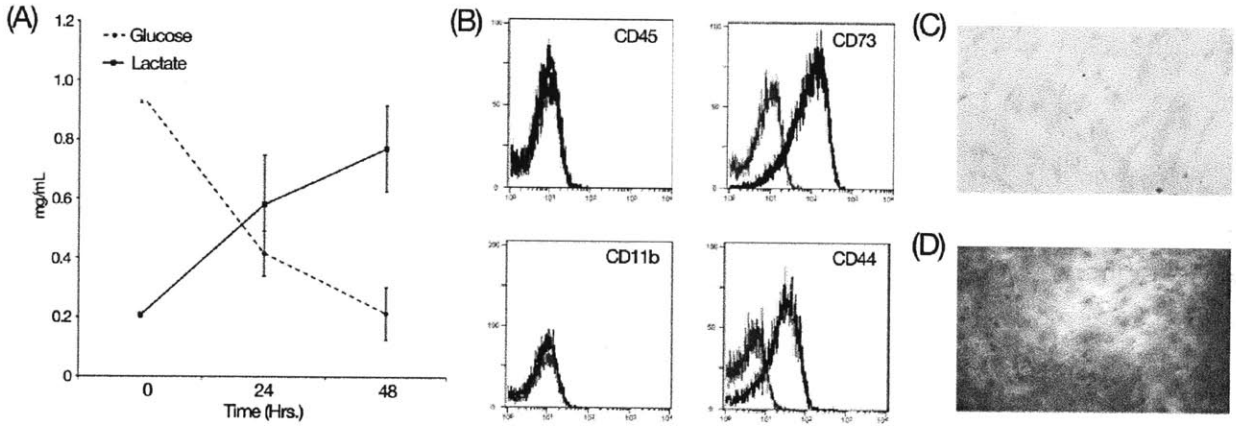


Figure 3.3.3 – MSCs retain their identity and differentiation potential after bioreactor operation. (A) Glucose consumption and lactate production over time of perfusion show cells maintain viability throughout a 48-hour time course in a bioreactor under flow. N=3. (B) Histograms of classical immunomarkers used to identify MSCs. A phenotype of $CD44^+$, $CD45^-$, $CD11b^-$, $CD73^+$ surface expression is preserved after a 48-hour bioreactor perfusion. Analysis was performed on a device seeded with 200 x 10^6 cells exposed to an ultrafiltrate flow rate of 10 mL/min. Black histogram is a stained sample and grey histogram is isotype control. N=2 (C) Microscopic images of Alizarin Red staining of MSCs directly after perfusion. Negative staining confirms that cells taken directly from the device showed no signs of osteogenic differentiation. (D) Alizarin Red stain of MSCs isolated from the device and differentiated for three weeks in osteoinductive medium demonstrating MSCs still retain the potential to become an osteoblastic lineage after device operation. N=2.

We also evaluated osteogenic differentiation of MSCs from the bioreactor. Cells immediately taken from the device showed no osteogenic differentiation as measured by Alizarin Red staining for mineral content (**Figure 3.3.3d**). In addition, MSCs isolated from the device were also tested for their capacity to differentiate after incubation with osteoinductive factors. After three weeks of culture in osteogenic differentiation medium, MSCs stained positively for rich calcium deposits verifying the capacity to still produce osteoblastic progeny (**Figure 3.3.3d**).

3.3.4.4. Secretion of cytokines and growth factors by human MSCs is maintained in a hollow fiber configuration

Human MSCs are known to secrete a wide spectrum of molecules that naturally support hematopoiesis *in vivo*. We examined two classical, but distinct factors: interleukin (IL)-6 and vascular endothelial growth factor (VEGF). IL-6 is a pleiotropic cytokine that is essential for lymphocyte differentiation and antibody production [282]. During initial stages of inflammation, IL-6 has demonstrable systemic effects on epithelial cell regeneration [283], muscle contraction [284], lipolysis [285], and wound repair [286]. IL-6 was secreted by MSCs accumulated in the reservoir of the bioreactor circuit and was comparable to secretion in 2D tissue culture plates (**Figure 3.3.4a**).

VEGF is the quintessential growth factor involved in the stimulation of angiogenesis – the formation of new microvessels [287]. The release of VEGF by MSCs has been implicated in the therapeutic response associated with transplantation. VEGF appeared to plateau over time in the device while continuing to increase in a 2D culture plate (**Figure 3.3.4b**). These data suggest that the secretome of human MSCs can be preserved in a bioreactor culture platform.

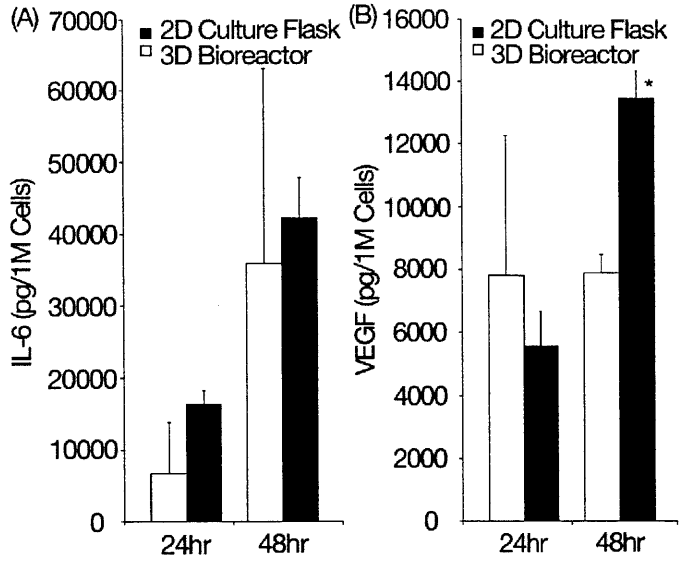


Figure 3.3.4 – MSCs secrete two well-known factors in a bioreactor. ELISA results of (A) Interleukin-6 (IL-6) and (B) Vascular Endothelial Growth Factor (VEGF) during a 48-hour bioreactor perfusion. Secreted factors were measured in the reservoir of the bioreactor circuit from 200 x 10^6 cells exposed to an extraluminal flow rate of 10 mL/min. The results are compared to MSCs seeded in 2D tissue culture plates; $^*p < 0.05$. All data is normalized to cell mass. N=3.

3.3.4.5. The effluent of MSC-laden devices increases the production of IL-10 from PBMCs

Our prior experiments demonstrated that human MSCs retain their viability/identity in hollow-fiber bioreactors and that bioactive molecules can be isolated from the effluent of the device. As a proof-of-concept, we lastly explored if human MSC secreted factors retained potency when considered for therapeutic applications. Human MSC secretions are reported to have paracrine, anti-inflammatory effects on immune cells. We developed an *in vitro* potency assay of human MSC secretions that involves the incubation of conditioned medium (MSC-CM) with human PBMCs in the presence of LPS as a model, pro-inflammatory agent (see Appendix for a detailed discussion of this method of potency assessment). When employed, MSC-CM leads to the upregulation of IL-10 cytokine release, which is directly associated with the therapeutic effect of MSCs in immune-mediated diseases [146]. We collected medium from the MSC bioreactor and executed our PBMC potency assay without concentration of MSC-CM. MSC-CM from the device showed a significant increase in IL-10 production from LPS-stimulated PBMCs as compared to 2D controls over time (**Figure 3.3.5**).

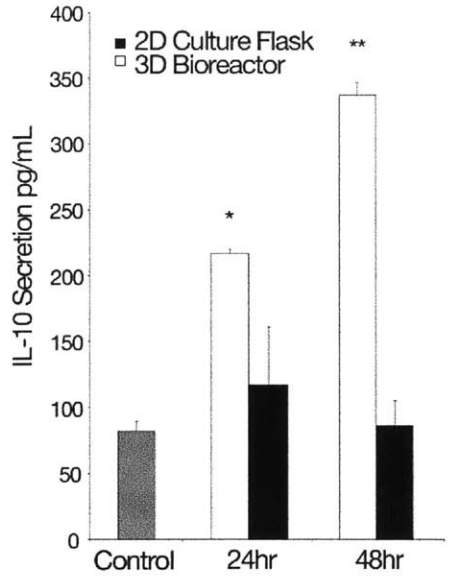


Figure 3.3.5 – MSC bioreactor effluents have enhanced anti-inflammatory properties. Upregulation of IL-10 in peripheral blood mononuclear cells (PBMCs) after exposure to unconcentrated medium harvested from MSCs cultured in 2D flasks or 3D bioreactors. PBMCs were incubated in the medium for 18 hours, after which, cells were stimulate with LPS for 5 hours. Supernatant was collected and measured for IL-10 through ELISA; $*p < 0.05$, $**p < 0.001$. Baseline results represent PBMCs incubated with unconditioned medium. N=3.

3.3.5. Discussion

Today, hollow fiber bioreactors provide a useful platform for the production of cell-derived factors with exploratory application in clinical settings [216, 221, 288, 289]. MSCs, in particular, provide an ideal cell type to use in bioreactors given the reported therapeutic potential of their secreted molecules [280, 281]. Furthermore, the fluidic environment in a hollow fiber bioreactor is a simplified representation of the niche that MSCs are naturally found within the bone marrow. MSCs are perivascular cells that are accustomed to being proximal to blood-flowing sinusoids *in vivo* [68, 74, 75]. To recreate this microenvironment, we have developed MSC coatings for hollow fibers found in continuous blood flow devices. Our findings demonstrate that MSCs retain their phenotype after culture in hollow fiber bioreactors and have enhanced anti-inflammatory secreted factors.

The first aspect of engineering human MSC coatings was to determine if the cells would adhere to PES fibers. Prior studies have reported that a wide of array of human cells are capable of growth on the fibers and that surface modifications have improved attachment for particular cells [290, 291]. We observed that MSCs did not require any surface modifications to adhere to PES fibers. Although the mechanism by which MSCs adhere to

PES fibers has not been evaluated, we speculate that extracellular matrix proteins found in the growth medium may serve as a temporary substrate for the cells to then lay down a more permanent matrix.

The transition from static two-dimensional tissue culture to a dynamic three-dimensional bioreactor introduces a number of new variables that may affect the phenotype and functional characteristics of cells. Such changes in substrate composition and shear stress have been used as tools to guide the differentiation of MSCs [292], although there has been no work regarding culture in a hollow fiber bioreactor. Our studies have shown that the cells retain viability for a period of at least 48 hours when placed in perfusion culture. Four characteristic MSC surface markers ($CD44^+$, $CD45^-$, $CD73^+$, $CD11b^-$) remained expressed at known levels when exposed to shear while adherent on the fibers. In addition, we demonstrated that MSCs do not differentiate within the device but retain the capacity to do so after bioreactor operation.

An essential component of this cell-based technology relies on the ability of MSCs to secrete soluble factors in a consistent fashion within this new culture platform. There are clearly numerous soluble factors that could have been chosen from the compendium of MSC secreted molecules. We chose VEGF and IL-6 as representative molecules from distinct protein families. Our proteomic analysis indicated secretion of both VEGF and IL-6, although the trends were distinct. IL-6 exhibited a continual upward trend while VEGF levels nearly reached a plateau within 24 hours. The dynamics of each secreted species should therefore be kept in mind if the end-goal of a MSC device is to harvest a particular molecule en masse.

Beyond the isolation of individual factors from MSCs, we sought to evaluate the therapeutic potential of the collected factors released by MSCs in this device. We employed a potency assay that we have developed to measure the anti-inflammatory cytokine release of LPS-stimulation human PBMCs after incubation with MSC-derived factors. Our study shows that MSCs continue to secrete factors that upregulate IL-10 production in PMBCs. As

opposed to the use of conditioned medium from MSCs cultured in conventional tissue culture flasks, an intermediate medium concentration step was not required before using in our potency assay which suggests that the unknown, causative agents for IL-10 upregulation are found at target concentrations in the device effluent at the given cell mass employed. We observed a time-dependent increase in potency, which alludes to the possibility that factors are accumulating in the circulating medium collection chamber that was sampled. A more comprehensive evaluation of the MSC secretome in this continuous flow environment may provide mechanistic insight into active agents that induce an anti-inflammatory phenotype in PBMCs.

Clinical precedence has been established for the therapeutic use of cellular hollow-fiber devices in acute liver and kidney injury, whereby epithelial cells have been used in these extracorporeal devices as an artificial method of organ support [221, 293]. Our therapeutic approach deviates from these predicate devices by delivering cytoprotective and immunomodulatory molecules rather than attempting to recapitulate the function of an injured organ. The results from this study support the use of MSC-based devices for indications that require an increase in IL-10, which include a number of immune-mediated conditions [294]. This platform can also be viewed as highly adaptable and not merely limited to IL-10 boosting therapies. For example, MSC secreted factors have also been shown to suppress TNF-α and IL-1 levels *in vivo* [42]. Since MSCs are retained outside of the body using this technology, it may also be possible to titrate therapeutic dosing by adjusting the cell mass in the device within an estimated maximum greater than one billion cells. This approach can offer more flexibility than cell transplantation that is limited by adverse events associated with transplanting too many cells. Ultimately, a MSC-loaded hollow fiber bioreactor may act as a combinatorial therapeutic delivery system for the treatment of inflammatory diseases.

4. Deriving Novel Protein Therapeutics Inspired by Bone Marrow Mesenchymal Stem Cell Secretions

4.1. Introduction

In the last chapter, Chapter 3, we saw that, consistent with previous results from our lab, MSC-CM possesses the molecular means for conveying significant therapeutic support in the context of acute kidney injury, a devastating inflammatory organ failure syndrome that is responsible for hundreds of thousands of deaths annually worldwide. A critical step in the direction of translation was also taken as we worked to scale-up the MSC bioreactor platform pioneered by my predecessors [41] to accommodate large animal testing and eventual human use. It will not be disclosed in much further detail in this thesis, but work is ongoing in this area, including large animal testing, and will continue as we push towards commercial development of this technology.

In this chapter, we will diverge from the topics of the previous chapters and embark on a journey inspired by the observations and discoveries made therein. As described in the first chapter of this thesis, we first began our investigation into the contents of MSC secreted factors as an extension of our work in MSC therapy: we were interested in better understanding the mechanisms of action of MSCs through the characterization of their secreted factors. Nevertheless, we quickly recognized the potential value of our discovery from the perspective of protein drug discovery, and instead sought to make best use of this collection of therapeutic factors as a source of novel candidate therapeutic molecules.

The successes we enjoyed as a result of this departure will be described in this chapter. Without question, while we did pursue a divergence from our previous work, it was convergence of many aspects of the previous work of ours and others that made this new direction possible. In particular, the development of a biologically relevant potency assay using primary human peripheral blood mononuclear cells with a measurable, reproducible

and positive output, relevant to human disease: this was one of the critical turning points that allowed us to venture down this path. We have since used this potency assay throughout our work (as described in earlier sections and outlined in detail in the Appendix), but many of the experiments that validated its predictive power *in vivo* and its demonstrated reproducibility were performed while pursuing the work described in this chapter.

Before I describe the results of this work, I would like to first introduce several concepts and review the current literature to provide some background for the problem we sought to solve.

4.1.1. Dissecting Conditioned Medium to Identify Mediators of Cellular Function

The concept of probing the contents of secreted molecules from cells is not new. In fact, many of the most important cytokine discoveries came out of experiments and observations not unlike those we describe in the previous chapters. However, the methodologies employed to dissect secreted contents of cells have changed over the past decades, and later in this chapter we will go into the details of how we developed a new approach to probing the contents of cell secretions that leverages contemporary technologies. Nevertheless, the standard, and widely accepted approach to dissecting a heterogeneous mixture of molecules involves separation and identification, usually entailing chromatography followed by mass spectrometry, respectively. Many of the examples of conditioned medium dissection have leveraged these methods to identify many powerful molecules involved in a wide variety of cellular processes. **Table 4.1.1** highlights just a few of the many examples of proteins discovered by taking this approach.

Table 4.1.1 – Cytokines and growth factors discovered in conditioned medium

Protein	Type of CM	Potency Assay(s)	Chromatography	Other Separation	Liters of CM	Ref
GCSF	Mouse lung stimulated with LPS	Mouse myelomonocytic leukemic cell (WEHI-3B) differentiation	Salting out Phenyl-sepharose Bio-Gel P60 Phenyl-silica HPLC TSK gel HPLC	N/A	3	[295]
LIF	Mouse L929 fibroblast cells	Mouse myeloid leukemic cell (M1) differentiation	DEAE-cellulose (anion exchange) Sephadex G-200 Phenyl-sepharose C_{18} Hydrophobic Support TSK gel HPLC	Ammonium-sulfate precipitation	93	[296]
IL-2	PHA-stimulated human lymphocytes	T-cell proliferation as determined by [^{3}H]thymidine	DEAE-cellulose (anion exchange) Ultrogel HPLC	Ammonium-sulfate precipitation Dialysis Isoelectric focusing PAGE	1.5	[297]
MCP-1	THP-1 cells stimulated with LPS, silica and hydroxyurea	Monocyte chemotaxis	DEAE-cellulose (anion exchange) Heparin sepharose Sephacryl S-200 gel HPLC Cation exchange Reverse phase HPLC	Dialysis	21	[298]
SCF	Buffalo rat liver cells (BRL-3A)	Mouse hematopoietic stem cell proliferation MC/9 mouse mast cell proliferation as determined by [^{3}H]thymidine	DEAE-cellulose (anion exchange) Ultrogel HPLC Agglutinin-agarose S-sepharose (cation exchange) Reverse phase HPLC	Dialysis PAGE	336	[299]
Interferon	Human leukocytes stimulated with the Newcastle disease virus	Interferon protection of MBDK bovine kidney epithelial cells when challenged with virus	Sephadex G-100 gel filtration Licrosorb RP-8 HPLC Licrosorb diol HPLC	Acid precipitation	10	[300, 301]

Biochemistry has taught us that there are properties of all molecules that allow them to be uniquely singled-out from a heterogeneous mixture. For example, size, charge, polarity and solubility are properties of all biomolecules that are commonly used for separation. Common separation strategies take advantage of the fact that very few molecules possess the exact same combination of chemical and physical properties – while some molecules

exhibit very similar charge and polarity, they might differ significantly in size, etc. The field of chromatography is dedicated to the development of many useful techniques and technologies to assist in separation. Once a molecule is successfully isolated using various levels of chromatographic separation, abundant modalities of mass spectrometry exist for detailed molecular analysis of the resultant isolate. New proteomic analytical laboratories, for example, have developed dedicated chromatography and mass spectrometry platforms for identifying fine molecular modifications among proteins, revealing biological changes as subtle as post-translational modifications of single proteins out of mixtures of cellular extracts [302].

As it pertains to conditioned medium, these techniques, while tried-and-true, are often limited technically, depending on the material to be isolated and identified. As can be seen in **Table 4.1.1**, some molecules can be isolated from only a few liters of conditioned medium, but more elusive molecules require hundreds of liters, which requires costly infrastructure for large-scale cell growth and conditioned medium production [299]. Given the value of certain molecules, especially those that can be developed as recombinant protein therapeutics [303], this investment can pay off, but large-scale characterization of the cell secretome is cost prohibitive with these methods. As we show later, genomic technologies, when leveraged correctly, can be powerful new tools for tackling this same problem, and offer compelling advantages in terms of cost and scale.

4.1.2. Choosing an MSC-Relevant Measure of Potency

As is clear in **Table 4.1.1**, every separation strategy requires a potency assay to trace the activity of fractions of conditioned medium. For the results of the separation to be relevant, the potency assay must be based on realistic biological phenomena, and the most valuable potency assays are those that predict efficacy of isolated factors as modifiers of disease processes. The most biologically relevant potency assays are low throughput, and often entail disease modeling in animals. Often, as the potency assay is adapted to accommodate scaling constraints of higher throughput, the biological relevance suffers –

higher throughput assays usually require the use of *in vitro* methods that are more easily reproducible and less complicated than whole-organism studies [304]. In order to perform a meaningful screen, therefore, a balance must be struck between throughput of the potency assay and the relevance of the output. High throughput screening (HTS), used for decades as a major modality of drug discovery, is often severely limited by the relevance of the assays developed to accommodate the hundreds of thousands or millions of molecules screened [305]. Add to that the fact that most screens are only testing single concentrations of each compound in a library, and it is no wonder HTS approaches have produced underwhelming results [306]. Ideally, the libraries of compounds screened would be smaller and more concentrated with relevant potential hits, which could accommodate medium- or low-throughput assays that preserve biological relevance [307-309]. As will be seen later in this chapter, using gene expression analysis, we were able to generate a highly enriched library of potential protein therapeutics that allowed us to use a medium-throughput potency assay that preserved biological relevance *in vivo*.

Generally speaking, for an *in vitro* medium-throughput assay, primary cells isolated from human subjects are superior to immortalized cell lines and primary cells from animals as they mimic as closely as possible the behavior of human biology. Unfortunately, few diseases can be easily modeled with primary human cells that are amenable to isolation and manipulation with minimal perturbation of natural function. This is likely why human peripheral blood mononuclear cells are used often in potency assays, as this fraction contains many different circulating immune cells, including monocytes, neutrophils, NK cells, T cells and B cells, which is a sufficient composition of cells to accurately model immune activity in whole human blood [310]. For our purposes, we sought a potency assay that would not only be as biologically relevant as possible, but also would reveal an output that could be used to assess MSC activity. Fortunately, we were able to adapt an assay developed by Németh and co-workers [146] to suit our own purposes [258] (see Appendix), that leveraged PBMCs to measure the anti-inflammatory activity of MSC-CM, with IL-10 as the measure of potency.

Németh and colleagues demonstrated that MSCs, when transplanted in the context of murine cecal ligation and puncture sepsis, are effective at protecting animals from death by a contact- and prostaglandin-mediated upregulation of IL-10 in macrophages [146]. Based on the apparent importance of IL-10 in the MSC-mediated protection of mice with sepsis, and based on our observation that IL-10 is upregulated in animals treated with MSC-CM, we hypothesized that by modifying Németh's assay and replacing macrophages with PBMCs (the primary population of cells first exposed to MSC-CM upon i.v. administration), we might see a similar increase in IL-10 secretion in PBMCs incubated with MSC-CM and stimulated with LPS. As will be described later, we indeed observed a significant increase in IL-10 production from PBMCs incubated with MSC-CM and then stimulated with LPS, providing for a reproducible potency assay.

In addition to having been shown as an important intermediate in MSC and MSC-CM therapy, IL-10 is a pleiotropic cytokine that as a measure of potency has profound therapeutic implications. IL-10 is widely viewed as the archetypical anti-inflammatory cytokine because of its capacity for suppressing early-phase inflammatory cytokine production in various immune cells [265]. While it has a systemic half-life of about two hours [311, 312], by suppressing IL-1, IL-6, IL-12 and TNF-α from DCs, macrophages and monocytes during acute-phase inflammation, IL-10 is capable of providing significant support during cytokine storm [313, 314]. IL-10 has been shown to suppress antigen presentation by DCs and mediate tolerance [315, 316], regulate T cell differentiation via modulating production of T helper cell type 1 cytokines, IL-2 and IFN-γ, and helper type 2 cytokines IL-4 and IL-5 [317], and is critical for the effector function of regulatory T cells [318]. Given its broad ability to suppress inflammatory processes, IL-10 has been explored as a therapeutic in a variety of animal disease models. It was first observed in IL-10 knockout mice that spontaneous enterocolitis formed, implicating the important role IL-10 plays in regulating colonic homeostasis and preventing the onset of IBD [319]. Multiple studies demonstrated that exogenous IL-10 administration could prevent onset of colitis in a variety of models of colitis, but only when given prior to disease onset [320, 321]. Later studies showed that genetically modified L. lactis that constitutively expressed IL-10 could be used as a treatment for murine

colitis [322]. Other animal disease models in which IL-10 has been shown to have an effect include diabetes mellitus [323], experimental autoimmune encephalitis [324], pancreatitis [325], and various models of arthritis [326, 327]. Based on the encouraging results of these animal studies [328], IL-10 has also been tested in humans, with efforts primarily led by the former Schering-Plough and DNAX Research Institute. **Table 4.1.2** summarizes diseases in which IL-10 has been tried therapeutically [328].

Table 4.1.2 – Human trials of interleukin 10 therapy

Indication	Trial Progression	Patients Treated	Study Conclusions	Ref
Crohn's	Phase I Phase II	Mild to moderate Therapy refractory CD Curative resection	IL-10 is safe No lasting remission with systemic administration High dose side effects: anemia and headache	[329-332]
Rheumatoid Arthritis	Phase I Phase II	Adult RA patients	IL-10 is safe 63% of patients achieved ACR 20 response compared to 10% with placebo with an IL-10 dose of 20 μg/kg 3 times per week; higher ACR responses were not observed	[333, 334]
Psoriasis	Phase I Phase II	Adult patients with moderate to severe psoriasis	IL-10 well tolerated In double-blind, placebo controlled studies, modest trend towards improvement, no lasting remission	[335-337]
Chronic Hepatitis C	Phase I	Adult patients with HCV	IL-10 prevents liver fibrosis Liver enzymes decreased, indicating lower level of liver injury during chronic infection	[338, 339]
HIV	Phase I Phase II	Adult HIV patients	IL-10 is well tolerated IL-10 does not provide immunological or virological benefit	[340]

Unfortunately, the clinical trial results of IL-10 therapy have not lived up to expectations. Many assert that this can be attributed to the relatively short half-life of IL-10 [328], especially as a treatment for diseases that require systemic administration. However, it is clear from the animal and human studies of IL-10 that it exerts powerful effects on inflammatory processes. IL-10 boosting strategies, therefore, that enhance endogenous production might allow for successful use of IL-10 as an intermediate anti-inflammatory molecule without the shortcomings of exogenous delivery. Since MSC-CM has been shown to boost IL-10 *in vitro* and *in vivo*, we decided it would be an excellent proxy for therapeutic activity, and an excellent potency indicator during CM stratification.

We subsequently used IL-10 as our biomarker for therapeutic activity, and as the next section will describe, successfully broke down the contents of MSC-CM to elucidate novel IL-10 boosting agents expressed by MSCs. As a final proxy for activity, we also wanted to confirm that the IL-10 boosting that we measured *in vitro* was relevant *in vivo*, so we chose to use a mouse potency assay involving the same pro-inflammatory ligand as we used for the *in vitro* potency assay, lipopolysaccharide (LPS). LPS molecules constitute the majority of the outer coating of gram-negative bacteria, and signal through toll-like receptor (TLR) 4 in most mammalian species to activate pro-inflammatory transcription factors such as NFκB [341, 342]. This pathway is highly conserved across species as a first level of defense against gram-negative microbial infection. In animals, LPS is a classical stimulant of acute-phase inflammation, and at a high dose precipitates cytokine storm and shock [343]. We chose to investigate this disease model not only as a proxy for therapeutic activity of isolated MSC factors in the context of septic shock, but we also reasoned that LPS-mediated signaling could yield a response relevant to other diseases as it does involve extensive communication of pro- and anti-inflammatory early-phase mediators that have also been shown to play major roles in other diseases of inflammation and autoimmunity [344, 345]. Future work will entail further exploration of the anti-inflammatory utility of the factors discovered in other diseases.

4.2. Enriched Protein Screening Reveals MFAP5 and PENK as Novel Cytokine Modulators Inspired by Human Mesenchymal Stem Cell Secretions

4.2.1. Abstract

Protein therapeutics comprise a major fraction of all administered drugs. Unfortunately, discovering new protein therapeutics is a tedious process that relies on years of detailed research into the nature of biological pathways to identify disease-modifying molecules.

Open-ended screening of proteomic libraries is cost prohibitive and intractable since the majority of the human proteome has not yet been synthesized. Here we present a new method to rationally screen for protein therapeutics that uses bioinformatics guided by enriched genomic datasets in combination with potency assay testing. We leveraged this method to identify four novel proteins with previously unknown anti-inflammatory properties expressed by human bone marrow stromal cells, two of which provided a significant survival benefit to mice challenged with lethal endotoxemic shock. Using this enriched protein screening approach, we achieved a remarkable hit rate of 18% with exceptional efficiency of time, expense, and labor compared to current methods of protein discovery.

4.2.2. Introduction

The market for protein therapeutics is growing faster than for any other drug class [346, 347]. Sales last year were predicted to make up nearly one third of all drug revenues and exceed $90B [348-350]. Despite the rapid growth, discovering new protein drugs can be prohibitively expensive: from 2007-09, the pharmaceutical industry invested $70.6B into research and development, yet only 11 new protein therapies were approved by the FDA during that period [346, 347]. Discovery of new candidates has primarily relied on research into the biology of disease and selecting agents for intervention, an inherently inefficient process [351]. Open-ended screening approaches that assume less about the underlying biology are impractical because establishment of proteome libraries is costly and not all proteins synthesized in the human body can be made recombinantly [352]. These approaches are also weak because throughput constraints require assays to be scaled, often resulting in a loss of biological relevance and increased risk of false negatives [353]. New methodologies for identifying potential protein therapeutics that preserve biologically relevant outputs while circumventing mechanistic understanding upfront could disrupt current discovery strategies and offer powerful insights into novel therapeutic phenomena.

We report here a new methodology that leverages the high biological relevance of a low-throughput human peripheral blood mononuclear cell (PBMC) potency assay in concert with virtual candidate enrichment using gene expression profiling to identify potential protein therapeutics naturally expressed by bone marrow stromal cells (BMSCs). BMSCs are stem-like cells that are being explored clinically as a novel therapeutic for a variety of inflammatory and autoimmune disease [267]. The implementation of this methodology led, in a matter of months and for under $50,000, to the discovery of two novel proteins, microfibrillar associated protein 5 (MFAP5) and unprocessed proenkephalin (PENK), that when given to mice challenged with the classical TLR ligand and acute phase agonist LPS, reversed the cytokine storm and provided a significant survival benefit comparable with the gold-standard anti-inflammatory biologic, anti-TNF-α. The design of our approach (referred to as enriched protein screening, EPS) is shown and described in **Figure 4.2.1a.**

4.2.3. Materials and Methods

4.2.3.1. Statistics

Unless otherwise noted, all experiments were repeated in quadruplicate, and all data were assessed for significance using a paired, two-tail Student's T test.

4.2.3.2. Cell Culture and Conditioned Medium

BMSCs were isolated, purified, grown and characterized, and fibroblasts grown as described previously [41, 258]. All BMSCs were used at passage 2-5. The conditioned medium was also collected and concentrated as described previously [42]. For the $BMSC_{LPS}$ conditioned medium and cells used for gene expression analysis, BMSCs were grown to >80% confluence and rinsed twice with PBS. BMSC or fibroblast expansion medium supplemented with 1 μg/mL LPS (E. coli 0111:B4; Sigma, St. Louis, MO) was then added to the cells for 24 hours, at which time the cells were rinsed with PBS twice again

and incubated for an additional 24 hours with serum-free DMEM to produce conditioned medium. By convention, 1X refers to the concentration of conditioned medium achieved when 15 mL of conditioning medium was incubated in the presence of $2x10^6$ cells for 24 hours, collected, and concentrated to a final volume of 1 mL.

4.2.3.3. Peripheral Blood Mononuclear Cell Potency Assay

The assay was performed as before ([258]; see Appendix). Approval for the collection of blood from healthy volunteers was obtained from the Institutional Review Board of Massachusetts General Hospital. For the majority of the experiments, the potency assay was terminated at 5 hours for IL-10 analysis.

4.2.3.4. Gene Expression Analysis

Gene expression was evaluated using Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA). Array quality was assessed using the R/Bioconductor package [354]. All arrays passed visual inspection and no technical outliers were identified (n=3 arrays per cell type). Raw CEL files were processed using the robust multiarray average (RMA) algorithm [355]. To identify genes correlating with the observed phenotypic groups, we used limma [356] to fit a statistical linear model to the data and then tested for differential gene expression in the contrasts of interest: FB vs. BMSC; BMSC vs. LPS. Results were adjusted for multiple testing using the Benjamini and Hochberg (BH) method [357], and significance was determined using a False-Discovery-Rate cutoff of less than 1%. All genes identified to be upregulated in BMSCs compared to FBs and either expressed equally or upregulated in $BMSC_{LPS}$ compared to BMSCs were then analyzed by literature review for definitive evidence of production of a secreted protein by human cells to generate the list of genes reported here. Independent analysis conducted in collaboration with a second computational biology facility confirmed these same results with >95% overlap of secreted proteins identified via literature review.

4.2.3.5. Recombinant Protein Screen

Recombinant proteins were acquired from a commercial vendor (Abnova, Taipei, Taiwan). The proteins were diluted in PBS and added to the PBMC potency assay to achieve a range of final concentrations spanning ~1 μg/mL to ~0.01 ng/mL.

4.2.3.6. ELISAs and Western Blots

The ELISA kits used were provided by commercial vendors and were used according to the manufacturers' instructions (IL-10 in cell supernatants: BD, Franklin Lakes, NJ; LGALS3BP: Abnova, Taipei, Taiwan; IL-10 and TNF-α from animal serum: R&D Systems, Minneapolis, MN). To blot for GALNT1, MFAP5 and PENK, FB-CM, BMSC-CM and $BMSC_{LPS}$-CM were run out using protein gel electrophoresis (Pierce, Rockford, IL) followed by blotting using detection antibodies (Sigma, St. Louis, MO) applied at a dilution of 1:500 (GALNT1 and MFAP5) or 1:100 (PENK), and corresponding secondary antibodies (anti-rabbit for GALNT1 and MFAP5 and anti-goat for PENK) conjugated with HRP (Sigma, St. Louis, MO). For the LGALS3BP ELISA and the MFAP5 blot, 1X conditioned media were used, and for the GALNT1 and PENK blots, 20X conditioned media were used.

4.2.3.7. Mass Spectrometry

Mass spectrometry was performed at the Mass Spectrometry Core facility of the Beth Israel Deaconess Medical Center at Harvard Medical School as previously described [358]. 10X samples of BMSC-CM, FB-CM and $BMSC_{LPS}$-CM were initially separated by SDS-PAGE and bands were excised and trypsin digested for analysis via tandem LC/MS/MS. The false discovery rate (FDR) for peptide identifications was ~ 1.5% and less than 0.5% for protein identifications.

4.2.3.8. In Vivo Mouse Assay

All procedures were performed in accordance with the animal rights policies of the Massachusetts General Hospital Subcommittee on Research Animal Care. For the sub-lethal LPS model, eight week old female BALB/cJ mice (n≥3) (Jackson Laboratories, Bar Harbor, ME) were administered an initial dose of either vehicle or one of the experimental therapies IP: 200 μL of saline (vehicle), or 3 μg of protein (e.g. GALNT1, LGALS3BP, MFAP5 or PENK) diluted in 200 μL of saline, or 1 mL of 1X BMSC-CM. 16 hours later, the mice received a second dose of either vehicle or therapy in conjunction with a dose of 100 μg of LPS (E. coli 0111:B4; Sigma, St. Louis, MO) diluted in physiological saline. 48 hours later, the mice were sacrificed and tissue and blood were collected for analysis. The serum was tested for the presence of IL-10 and TNF-α via ELISA and the lungs, livers and kidneys of the animals were preserved for hematoxylin and eosin staining. For the lethal LPS model, eight week old female BALB/cJ mice (n≥5) were co-administered a lethal dose of LPS (350 μg LPS in 100 μL physiological saline) and either vehicle (negative control), 5 μg anti-TNF-α (positive control; R&D Systems, Minneapolis, MN), 4 μg MFAP5 diluted in 100 μL of physiological saline, or 4 μg of PENK diluted in 100 μL of physiological saline. The mice were monitored for survival for seven days (168 hours).

4.2.4. Results

We began this study with the development of an *in vitro* potency assay based on primary human PBMCs to evaluate the bulk anti-inflammatory activity of BMSC-CM (**Figure 4.2.1b**). We observed that LPS-stimulated production of IL-10 by human PBMCs increased significantly when they were incubated with BMSC-CM compared to vehicle control (**Figure 4.2.1c**). The upregulation of IL-10 release was time- and dose-dependent (**Figure 4.2.2a-b**) and was induced by protease-sensitive constituents of BMSC-CM (**Figure 4.2.2c**). To identify and purify individual factors responsible for this IL-10 response, we first attempted liquid chromatography (LC) and mass spectrometry (MS) analysis. We

successfully identified regions of activity with LC separation based on size and charge, but MS failed to elucidate specific factors even after concentrating and purifying BMSC-CM from >1x10^9 cells (**Figure 4.2.3a-b**). This led us to seek an alternative approach to screening BMSC secreted factors that would be more time, labor, and cost effective.

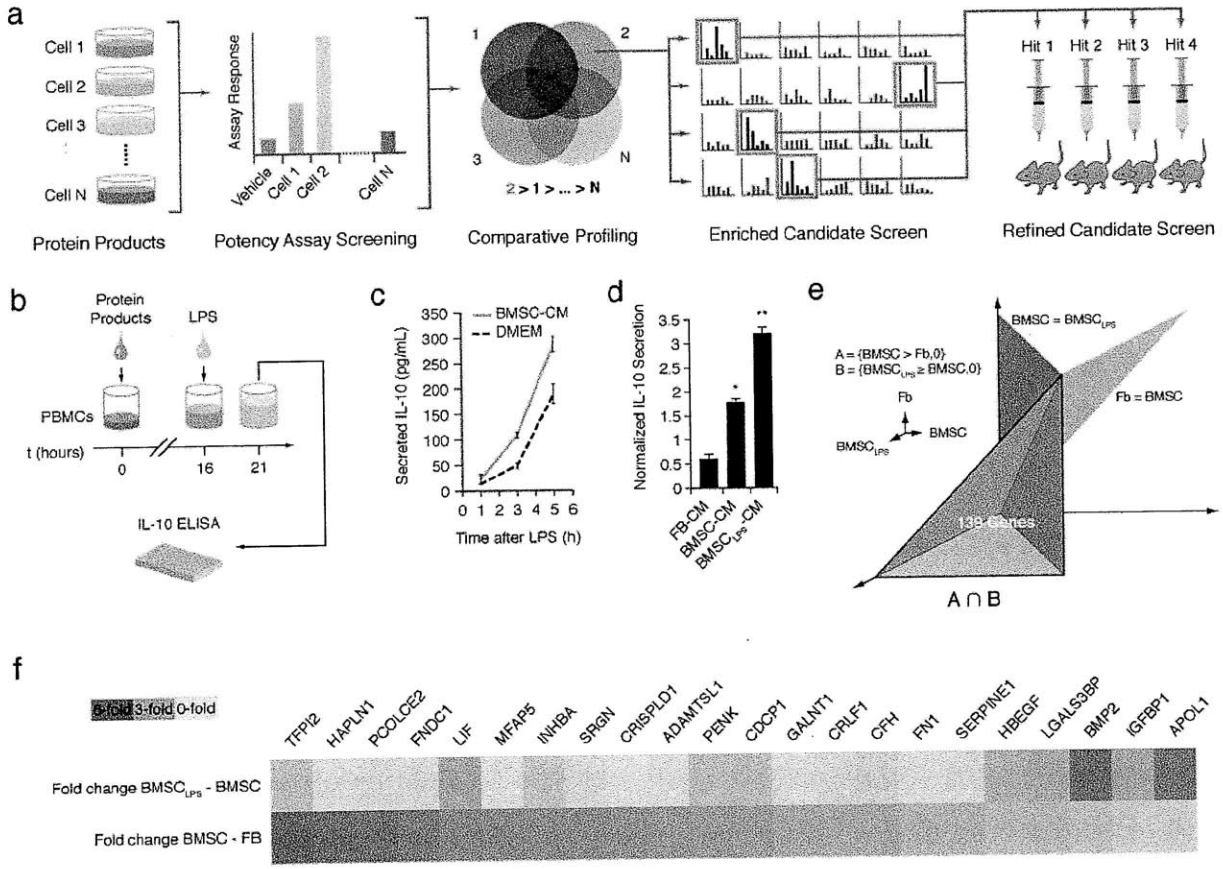


Figure 4.2.1 – Enriched protein screening (EPS) of BMSC-CM: approach, assay development and gene expression profiling. (a) Generalized schematic of EPS methodology. Protein products are derived from various cell types in the form of conditioned media and screened for activity in an *in vitro* potency assay. Based on the activity of the conditioned media from the cells, hierarchical comparative gene expression profiling is performed to select for genes uniquely upregulated in the cell type with the highest activity in the potency assay. Recombinant protein products of the enriched gene list are then screened in a potency assay and the candidates with the highest activity are assessed for activity *in vivo*. (b) The *in vitro* potency assay for this study. This assay entails incubating primary human peripheral blood mononuclear cells (PBMCs) in the presence of protein products (e.g. conditioned medium from a cell) for 16 hours, followed by stimulation of the PBMCs with LPS for five hours, and measurement of IL-10 secretion into the supernatant via ELISA. (c) Time course of IL-10 expression from PBMCs when incubated with either bone marrow

stromal cell conditioned medium (BMSC-CM) or unconditioned medium (DMEM) in the potency assay. (d) Comparison of potency assay activity of conditioned medium from normal human dermal fibroblasts (FB-CM), BMSCs (BMSC-CM) and BMSCs preincubated with LPS prior to conditioning ($BMSC_{LPS}$-CM). * $p < 0.001$ compared to FB-CM, ** $p < 0.001$ compared to BMSC-CM. (e) Schematic of the gene expression comparison scheme. Genes correlating with anti-inflammatory activity were selected by taking the intersection of the sets of all genes upregulated in BMSCs compared to FBs and all genes expressed equally or upregulated in $BMSC_{LPS}$ compared to BMSCs. (f) Gene expression profiling revealed 22 genes responsible for secreted proteins that were upregulated by BMSCs stimulated with LPS compared to BMSCs and FBs.

To circumvent individually screening the hundreds of proteins secreted by BMSCs [42, 359], we developed a comparative gene expression scheme that enabled us to rationally enrich for candidate proteins that contribute to the anti-inflammatory activity of BMSC-CM. To this end, we first sought to identify a "BMSC signature" in terms of BMSC genes that were associated with IL-10 upregulation in our potency assay. This led us to seek a comparative BMSC analog that was likely to express many similar genes as BMSCs, but lacked activity in the potency assay. We tested the CM from a similar stromal cell, normal human skin fibroblasts (FB), and found that FB-CM did not cause a significant increase in IL-10 expression in the potency assay (**Figure 4.2.1d**). Comparison of gene expression of BMSCs and FBs, yielded a list of ~500 genes uniquely upregulated in BMSCs. One additional, but essential comparative group, was then included to refine the "BMSC signature".

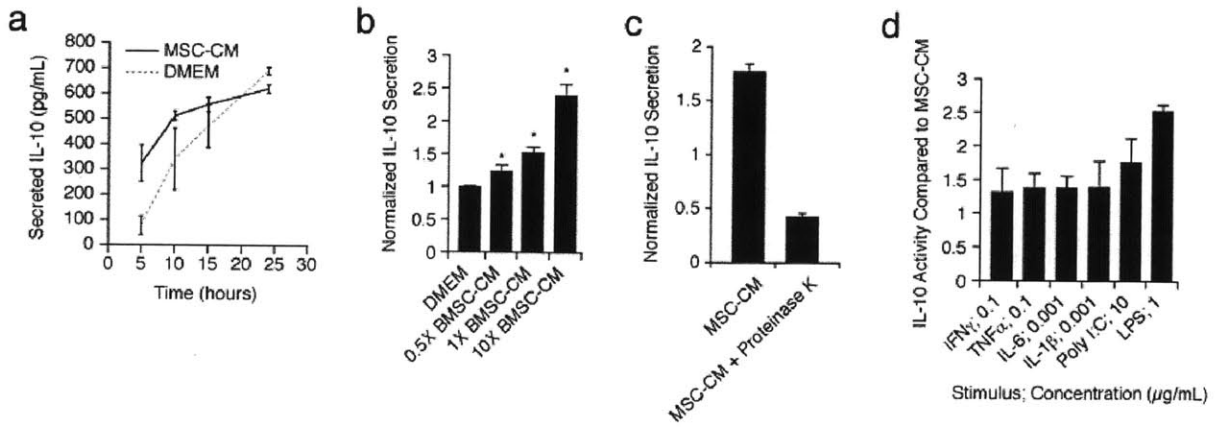


Figure 4.2.2 – Characterization of IL-10 assay and optimization of BMSC preconditioning. (a) Kinetics of IL-

10 secretion by PBMCs incubated with or without BMSC-CM and stimulated with LPS. (b) Dose response of the potency assay to increasing concentration of BMSC-CM. 1X CM was either diluted or concentrated further to generate the different concentrations. * $p < 0.001$ compared to DMEM. (c) Effect of proteinase K on the activity of BMSC-CM in the potency assay. (d) Effects of preincubating MSCs with various factors on the activity of BMSC-CM in the potency assay.

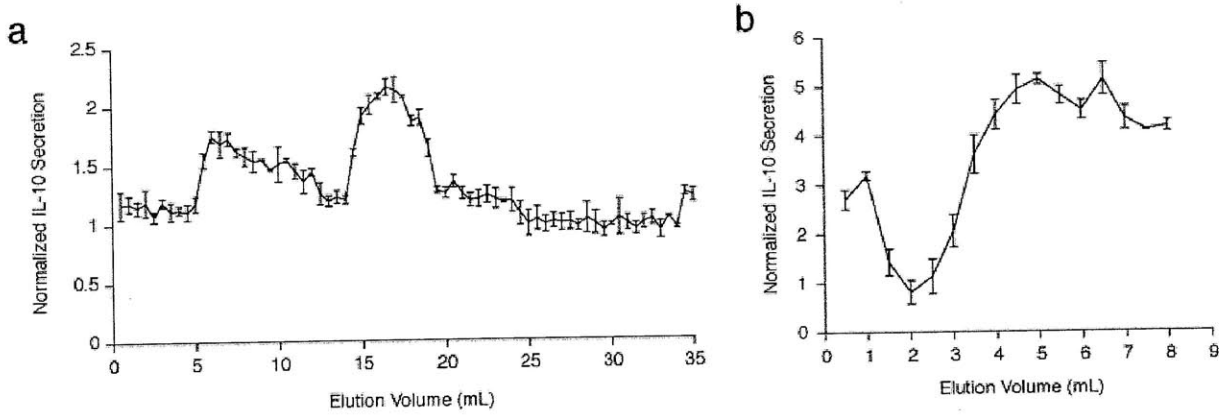
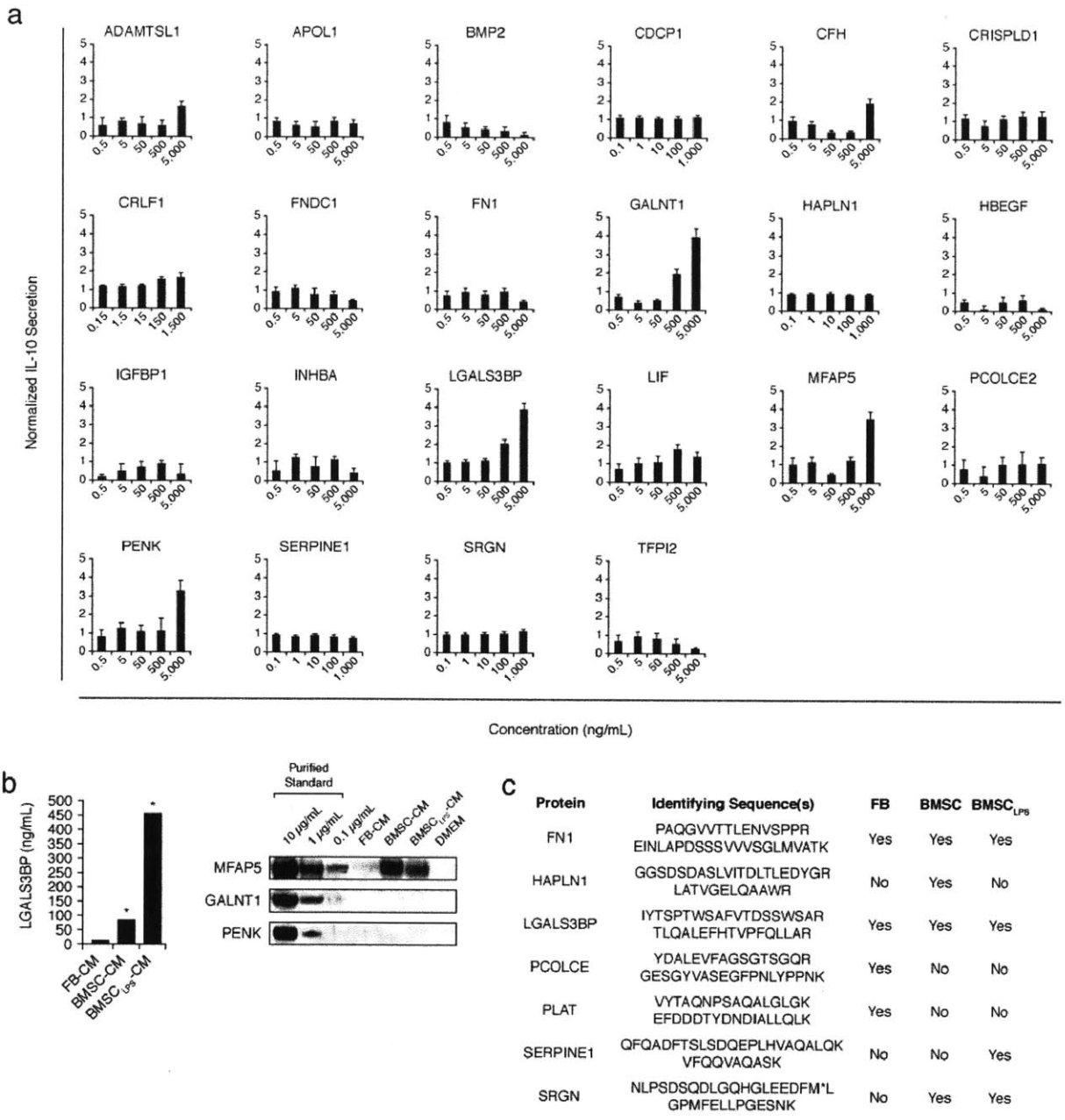


Figure 4.2.3 - Liquid chromatography of BMSC-CM. Chromatography was used to fractionate the BMSC-CM into 0.5 mL fractions and then the fractions were evaluated for IL-10 activity in the potency assay. (a) Fractions generated by size exclusion chromatography. (b) Fractions generated by anion exchange chromatography.

We perturbed the genomic signature of BMSCs by establishing conditions under which the activity of the BMSC-CM was enhanced. BMSCs are known to display a number of surface cytokine and toll-like receptors that have been implicated in their immunomodulatory phenotype [360]. We pre-stimulated BMSCs with a selection of the cognate ligands for these receptors for 24 hours prior to conditioning. We discovered that the BMSC-CM from cells that were pre-stimulated with LPS exhibited significantly higher activity in the potency assay (**Figure 4.2.1d and 4.2.2d**). We used this information to further refine our analysis by cross-referencing our list of BMSC-specific genes with genes that were maintained at the same level or significantly upregulated by BMSCs stimulated with LPS ($BMSC_{LPS}$) compared to BMSCs. This comparison revealed 139 genes (**Figure 4.2.1e**). We then conducted a literature search to determine which of these genes correspond to a secreted protein, yielding a highly enriched set of 22 genes (**Figure 4.2.1f**).



c

Protein	Identifying Sequence(s)	FB	BMSC	$BMSC_{LPS}$
FN1	PAQGVVTTLENVSPPR EINLAPDSSSVVVSGLMVATK	Yes	Yes	Yes
HAPLN1	GGSDSDASLVITDLTLEDYGR LATVGELQAAWR	No	Yes	No
LGALS3BP	IYTSPTWSAFVTDSSWSAR TLQALEFHTVPFQLLAR	Yes	Yes	Yes
PCOLCE	YDALEVFAGSGTSGQR GESGYVASEGFPNLYPPNK	Yes	No	No
PLAT	VYTAQNPSAQALGLGK EFDDDTYDNDIALLQLK	Yes	No	No
SERPINE1	QFQADFTSLSDQEPLHVAQALQK VFQQVAQASK	No	No	Yes
SRGN	NLPSDSQDLGQHGLEEDFM*L GPMFELLPGESNK	No	Yes	Yes

Figure 4.2.4 – Screening of the enriched recombinant protein candidates and assessment of BMSC secretion of the candidate proteins. (a) Enriched recombinant protein screen using the potency assay. (b) ELISA and western blotting of FB-CM, BMSC-CM and $BMSC_{LPS}$-CM for LGALS3BP, MFAP5, GALNT1 and PENK. * $p < 0.001$ compared to FB-CM. (c) Partial list of proteins contained in 10X FB-CM, BMSC-CM, and $BMSC_{LPS}$-CM detected by proteomic mass spectrometry.

We next assembled a purified recombinant protein library corresponding to the 22 candidate genes identified by our enrichment technique. We individually screened the

proteins in our potency assay using a range of physiologically-relevant concentrations and found that 4 of the 22 screened proteins successfully upregulated IL-10 secretion when present at ~100 nM concentrations (**Figure 4.2.4a**). To test whether BMSC-CM contained the four proteins, polypeptide N-acetylgalactosaminyltransferase 1 (GALNT1), galectin-3-binding protein (LGALS3BP), microfibrillar-associated protein 5 (MFAP5) and proenkephalin (PENK), we performed semi-quantitative Western blots for GALNT1, MFAP5 and PENK, and used a quantitative ELISA for LGALS3BP (**Figure 4.2.4b**). Clear bands were observed for MFAP5 and ELISA results showed LGALS3BP to be present at ng/mL concentrations in the BMSC-CM. GALNT1 and PENK were not present at detectable levels, even when the CM was concentrated 100-fold. This may be due to the fact that GALNT1 is only hypothesized to be secreted [361], and PENK is a pro-protein that is mostly processed into smaller peptide derivatives that might not react with the PENK antibody we used [362]. We also performed proteomic LC/MS on bulk BMSC-CM and identified several of the 22 proteins in our enriched library, including LGALS3BP (**Figure 4.2.4c**). Nevertheless, LC/MS failed to discern 18 of the 22 proteins (81%) from the enriched library, including GALNT1, MFAP5 and PENK. Taken together, these results demonstrate an unprecedented hit rate of protein discovery via EPS (18%), which is several orders of magnitude higher than traditional high throughput approaches that achieve *in vitro* rates on the order of 0.03-0.2% [363-365].

We then tested the four hits from the enriched recombinant protein screen for activity *in vivo* in animals challenged with a sub-lethal dose of LPS (**Figure 4.2.5a**). Significantly elevated serum IL-10 levels were observed in LPS-treated mice receiving BMSC-CM, GALNT1, MFAP5 and PENK compared to baseline vehicle control (saline), but no IL-10 response from LGALS3BP (**Figure 4.2.5b**). Serum TNF-α was also significantly suppressed in mice receiving BMSC-CM, LGALS3BP, MFAP5 and PENK, but not GALNT1 (**Figure 4.2.5c**). In addition, MFAP5 and PENK demonstrated superior TNF-α suppression compared to BMSC-CM. These results suggest that the different factors may influence different pathways, and it is conceivable that combinations of these factors could be used to treat multiple pathways in parallel.

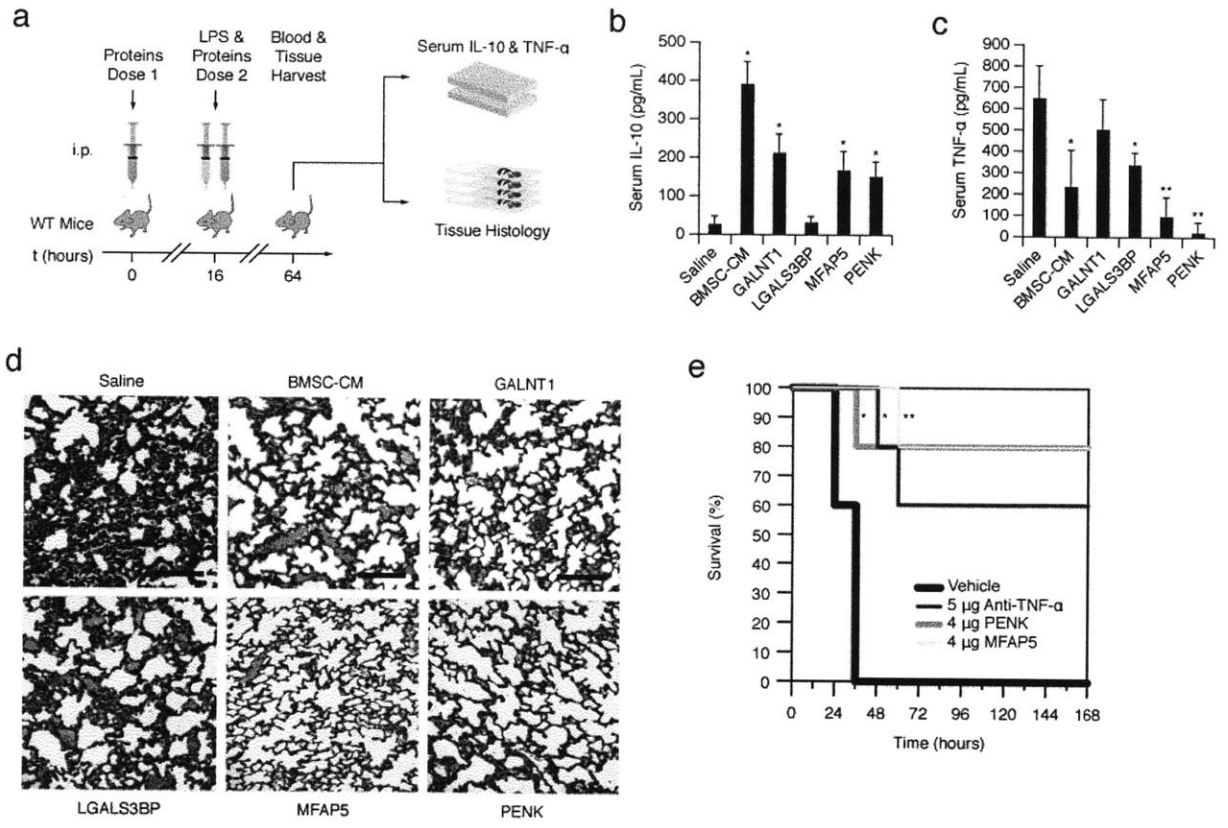


Figure 4.2.5 – *In vivo* hit screen and survival study. (a) Schematic of the *in vivo* LPS assay. Proteins were administered intraperitoneal (IP) at the concentration that elicited the strongest effect *in vitro*, followed by IP administration of LPS in conjunction with a second dose of the proteins 16 hours later. Two days after the combined LPS and second protein dose, the mice were sacrificed and assessed for changes in serum cytokines and tissue histology. (b) Serum IL-10 levels of BALB/cJ mice subjected to the *in vivo* LPS assay. * $p < 0.001$ compared to saline. (c) Serum TNF-α levels of BALB/cJ mice subjected to the *in vivo* LPS assay. * $p < 0.001$ compared to saline, ** $p < 0.05$ compared to BMSC-CM. (d) Representative micrographs of lung tissue from mice subjected to the *in vivo* LPS assay stained with hematoxylin and eosin. (e) Survival of mice subjected to a lethal dose of LPS i.p. (350 μg) concurrently with i.p. saline vehicle (bold dark blue line, n=20), 6 μg Anti-TNF-α antibody (thin dark blue line, n=5), 4 μg PENK (bold light blue line, n=5) or 4 μg MFAP5 (thin light blue line, n=5). * $p < 0.005$ compared to vehicle control, ** $p < 0.001$ compared to vehicle control. Scale bar = 200 μm.

The effects of cytokine modulation were apparent in the lung histology of the animals (**Figure 4.2.5d**). In vehicle treated animals, edema, inflammatory infiltrate and alveolar collapse were evident in all lung fields. MFAP5 showed superior lung protection,

preventing widespread inflammatory cell infiltrate and edema, thereby preserving the structure of the alveoli in the majority of the lungs. PENK, BMSC-CM and GALNT1 showed moderate protection with apparent inflammatory infiltrate, emphysematous changes and alveolar wall thickening, but no frank alveolar collapse. Based on these results, we then proceeded to test the two most promising proteins, MFAP5 and PENK in mice challenged with a lethal dose of LPS. Compared to anti-TNF-α, the gold-standard anti-inflammatory therapy for human use [366], both proteins exhibited similar protection and provided a significant survival benefit **(Figure 4.2.5e)**.

4.2.5. Discussion

Here we present two novel candidate anti-inflammatory protein therapeutics, both naturally expressed by BMSCs and identified using EPS. Neither have been previously reported to be secreted by BMSCs endogenously nor to contribute to the activity of BMSC therapy [256]. Moreover, neither have been previously demonstrated to possess potent anti-inflammatory activity. Proenkephalin A (PENK; a.k.a. PEA) is the precursor of the enkephalin opioid peptides and is proteolytically processed to yield Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Phe, Metenkephalin-Arg-Gly-Leu, enkelytin and PENK-derived peptides **(Table 4.2.1)** [367].

Table 4.2.1 – Peptide products of proenkephalin (PENK)

Name	Amino Acid Sequence	MW (da)
Met-enkephalin	Tyr-Gly-Gly-Phe-Met (SEQ ID NO. 14)	576
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu (SEQ ID NO. 15)	554
Peptide F (human)	Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Met-Glu-Pro-Glu-Glu-Glu-Ala-Asn-Gly-Ser-Glu-ne-Leu-Ala-Lys-Arg-Tyr-Gly-Gly-Phe-Met (SEQ ID NO. 16)	3846
Peptide E (human and bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu (SEQ ID NO. 17)	3157
Peptide B (human)	Phe-Ala-Glu-Ala-Leu-Pro-Ser-Asp-Glu-Glu-Gly-Glu-Ser-Tyr-Ser-Lys-Glu-Val-Pro-Glu-Met-Glu-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Phe (SEQ ID NO. 18)	3655

Cleavage of PENK by proteases takes place at dibasic, lysine-lysine, amino acid residues. It is expressed in specific sites in the brain and certain cells of the adrenal medulla and the immune system, which is increased at the gene expression level in response to inflammation [368]. The physiological relevance of the responsiveness of the PENK gene in these sites remains unclear. The concentration of PENK-derived peptides in the unstimulated bovine adrenal medulla exceeds 200 μg per gram of granule protein in secretory vesicles of chromaffin cells [369]. It is worthwhile to note that, except in the setting of meningococcemia, inflammation of the adrenal gland is a medical rarity. Peptides with antimicrobial activity but no known neuropeptide function have been identified in the adrenal medullary chromaffin cell discharge. They include enkelytin and peptide B from PENK [370]. Investigators have proposed that PENK contains neuropeptides, antibacterial peptides, and immune stimulatory peptides [371]. In addition to the neurohormone properties of PEAPs [372], numerous studies have demonstrated that PEAPs are secreted in various forms by cells in the immune system including T cells [373], neutrophils [374], and monocytes [375]. The effects of enkephalins on various activities of immune cells (chemotaxis, cytotoxicity, immunoglobulin synthesis), which bear opioid receptors, have been described. PEAPs have been shown to promote lymphocyte chemotaxis [376], suppress T cell activation when secreted by colon cancer cells [377], possess anti-bacterial properties [370, 378], and depending on the concentration, induce or suppress blastogenesis of human lymphocytes [379]. However, there have been no reports of PENK altering cytokine secretion from immune cells when administered directly in a purified form [380]. One study using PENK-deficient mice reported that T cells could produce IFN-γ and TNF-α but not IL-4 or IL-10 [381]. However, the study did not show a causal link between PENK and these cytokines. Overall, these studies might partially explain the immunomodulatory effects of PENK we have seen here, although a causal relationship between PENK and inflammatory cytokine modulation has not been previously demonstrated.

MFAP5 is an ECM glycoprotein localized to microfibrils and associated with elastin networks. It is a highly hydrophilic molecule consisting of two distinct domains: a cysteine-

free acidic N-terminal half, and a cysteine-rich basic C-terminal half. It has been found to have significant homology (57%) with MFAP2 (MAPG-1) [382], in particular with respect to the cysteine rich region. A series of 7 cysteines near to the center of both molecules precisely align when their sequences are compared, and the cysteine separation distances are highly conserved. Evidence of its relationship with the immune system is limited. MFAP5 binds fibrillin-1 and -2 at the C-terminus, as well as to other proteins containing EGF-like repeats [383]. It contains an RGD integrin-binding motif and has been shown to bind integrin [384]. MFAP5 has also been shown to interact with the Notch receptor pathway [385]. Conflicting accounts describe this interaction as either inhibitory [386] or activating [385], so the mechanisms by which MFAP5 binds Notch and instigates downstream signaling are yet unknown. Of note, to-date there have been no accounts of MFAP5 influencing immune processes, and its connection to human disease has not been established. It is interesting to note that the MFAP5 gene is located in the natural killer gene complex, and this location is highly conserved across species. Nevertheless, the strongest evidence that MFAP5 might play an endogenous role in immune function is seen in its relationship to cancer. It has been shown that serum MFAP5 (also known as MAGP-2) levels correlate with poor prognosis in patients with ovarian cancer [387]. In the same study, it was shown that tumor cells constitutively express MFAP5 *in vitro*, and could be the source of MFAP5 secretion *in vivo* [387]. In all, these data suggest that in addition to identifying potentially therapeutic molecules, our EPS approach has also revealed as yet unknown biology, both in terms of the implications of endogenous secretion of PENK and MFAP5 by BMSCs, and the anti-inflammatory role these two proteins might naturally serve *in vivo*. Judging by their size and the pattern of secretion by BMSCs and other cells, it is conceivable that MFAP5 and derivatives of PENK are *bona fide* cytokines, and may play important roles in the modulation of other immune functions besides inflammation [369, 388].

EPS has several advantages over traditional methods for protein drug discovery, making it a powerful new strategy for uncovering new therapies. First, by choosing a therapeutic output as the sole determinant of candidate activity (i.e. IL-10 upregulation), this approach

allowed us to screen without any hypotheses as to the nature or existence of receptor targets and bypass the years of research required to uncover intermediate ligand-receptor interactions. Second, EPS entails enrichment of a candidate population by design and thereby achieved an *in vivo* hit rate of 18% from our modest and inexpensive library of 22 recombinant proteins. This hit rate is several orders of magnitude higher than traditional HTS approaches that have been reported to exhibit *in vitro* rates on the order of 0.03-0.2% [363-365]. Finally, by using proteins as the basis for our screen, we ensured that all of our hits would be useful both as potential natural therapeutics, and as tools to uncover new anti-inflammatory pathways that have not yet been characterized.

We posit EPS can be applied to other cell types as well to reveal novel therapeutic molecules. EPS leverages the advantage of learning directly from the natural protein source to uncover new ways and means of modulating complex physiological processes to yield desirable outcomes. Also, we designed EPS to be modular such that the cell(s) and the assay(s) can be substituted to reveal genes that correlate with many diverse phenotypes. With the development of additional assays, enrichment of new recombinant libraries, and identification of novel potent secreted molecules, new classes of protein therapeutics could be discovered using EPS to treat difficult diseases in a natural and biologically relevant way.

5. Conclusions

The primary goal of this thesis was to build upon the current knowledge of MSCs and explore further the therapeutic utility of MSC secreted factors in a variety of inflammatory diseases. While pursuing this goal, my colleagues and I discovered that MSC secreted factors, independent of cell transplantation, were capable of providing significant protection to rats subjected to acute kidney injury. We found that the concentration of CM and dose regimen used provided a superior survival benefit and protection compared to other studies of MSC transplantation and MSC-CM administration in AKI. This finding was consistent with the work of my predecessors, and further suggested the general utility of MSC-CM. From these studies, we are confident in concluding that the mechanisms of MSC-CM therapy must share similarities in FHF and AKI, and could very well provide for significant support in other diseases with comparable underlying hallmarks of pathophysiology.

We also set out through the course of this thesis to begin to develop the original blood-contacting platform prototyped in our lab for clinical use, and make vertical advances towards successful translation. By seeding MSCs in standard hollow-fiber bioreactors (repurposed dialyzers) and subjecting these devices to conditions that mimic clinical operation, we were able to confirm that MSCs adhere and grow on polyethersulfone hollow fibers, are metabolically normal, do not differentiate during operation, and produce considerable and measurable amounts of proteins that are collectively therapeutically potent. This information will prove useful as we continue to test this device in large animals in preparation for human clinical trials.

In the final part of this thesis, we expanded in an orthogonal direction from our previous work, and began to regard the MSC-CM as a valuable source for mining potential protein therapeutics. Previous attempts to accomplish the same have provided valuable insight into the soluble mechanisms of MSC therapy, however never before had novel protein mediators been discovered to be secreted by MSCs. By developing a new methodology

for enriching the library of secreted factors from MSCs via comparative gene expression, we were able to identify multiple novel proteins that exhibit potent anti-inflammatory effects on human blood cells and in mice. Based on the potency of these molecules *in vitro* and *in vivo*, we are planning to pursue clinical translation with the goal of developing new protein therapeutics for inflammatory and autoimmune disease.

In all, the contents in this thesis have confirmed the findings of many groups that MSCs are extraordinary cells with meaningful biological and therapeutic implications. Continued efforts to optimize transplantation of the cells to maximize the conveyance of secreted factors holds great promise for the use of MSCs as a direct injectable in a variety of chronic inflammatory and autoimmune diseases. In addition, the development of new means for quickly administering MSC secreted factors in the context of acute disease, where the kinetics of engraftment and potential side effects of large doses of MSCs make transplantation unfavorable, will continue to expand the possible diseases in which MSCs might make a therapeutic difference. Finally, if our method for mining therapeutic cells for potential protein therapeutics can serve as the basis for new protein therapeutic discovery platforms, great advances are possible in the field of protein drugs, hopefully allowing for better therapies to be developed based on the natural processes of cellular communication. Together, these findings and discoveries hold great promise in contributing to better therapies for inflammatory and autoimmune disease, and will hopefully reveal new, as yet undiscovered facets of disease pathophysiology and resolution.

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7. Appendix

7.1. A Mesenchymal Stem Cell Potency Assay

7.1.1. Abstract

Mesenchymal stem cells (MSCs) are capable of modulating the immune system and have been used to successfully treat a variety of inflammatory diseases in pre-clinical studies. Recent evidence has implicated paracrine signaling as the predominant mechanism of MSC therapeutic activity. We have shown in models of inflammatory organ failure that the factors secreted by MSCs are capable of enhancing survival, downregulating inflammation, and promoting endogenous repair programs that lead to the reversal of these diseases. As a marker of disease resolution, we have observed an increase in serum IL-10 when MSC-conditioned medium (MSC-CM) or lysate (MSC-Ly) are administered *in vivo*. Here we present an *in vitro* model of IL-10 release from blood cells that recapitulates this *in vivo* phenomenon. This assay provides a powerful tool in analyzing the potency of MSC-CM and MSC lysate, as well as characterizing the interaction between MSC-CM and target cells in the blood.

7.1.2. Introduction

Bone marrow mesenchymal stem cells (MSCs) are resident non-hematopoietic progenitor cells that possess potent immunomodulatory abilities [99, 126, 128]. Allogeneic MSC transplants have been used to successfully treat hematological [13, 128], cardiovascular [108, 389], as well as neurological [390, 391] and inherited diseases [37, 392] in preclinical studies, and allogeneic transplantation of MSCs has been approved as a clinical treatment for severe GvHD [393]. Recent studies involving MSC transplantation have revealed that the therapeutic activity of these grafts is independent of differentiation, and paracrine

interactions of MSCs with tissue and immune cells provide the majority of therapeutic benefit [41, 42, 281, 394, 395].

We have found administration of concentrated MSC conditioned medium (MSC-CM) or MSC lysate (MSC-Ly) can reproduce the effects of a MSC graft *in vivo* and significantly increase serum IL-10 levels in two animal models [41, 42]. IL-10 is a well-known anti-inflammatory cytokine that can inhibit the secretion of pro-inflammatory cytokines and protect cells from apoptosis and necrosis in the context of acute inflammation [146, 265, 273]. We have determined that MSC-CM and lysate also substantially enhance IL-10 secretion by peripheral blood mononuclear cells (PBMCs) *in vitro*, and have developed an assay based on this discovery. This assay allows for rapid and reproducible assessment of the potency of MSCs and MSC molecular products in a manner relevant to animal and human testing of cell therapy.

Conditioned medium and cell lysate are derived from human MSC cultures and can subsequently be used for both *in vitro* and *in vivo* experimentation (see **Figure A1**). Below we provide methods for an ELISA-based IL-10 assay for *in vitro* testing, a powerful tool to aid in determining the efficacy and underlying paracrine mechanisms of MSC transplants and MSC-based therapies.

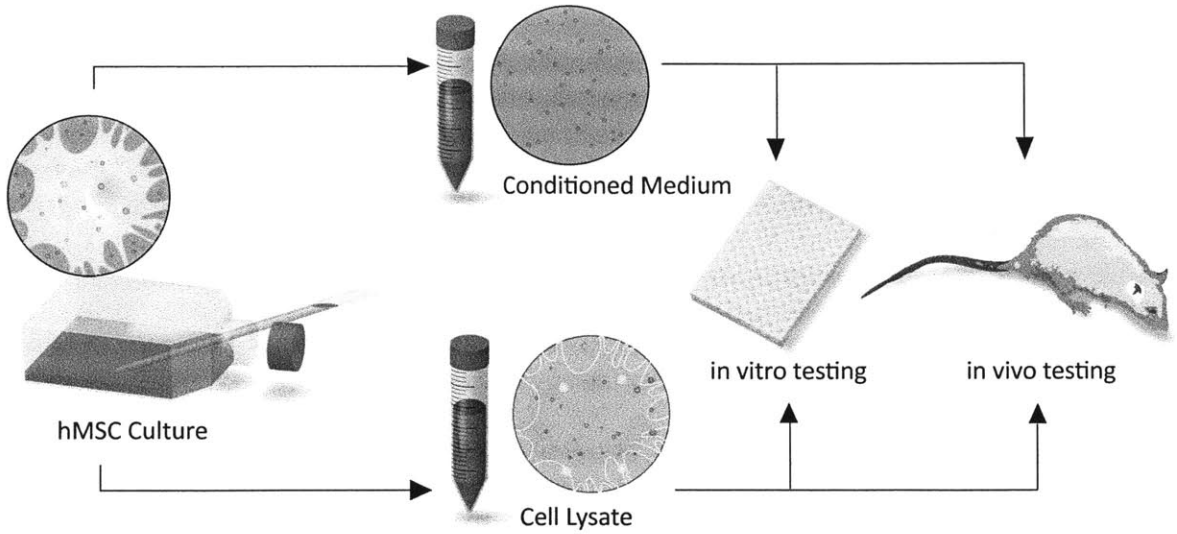

Figure A1 – Schematic of in vitro and in vivo potency tests of MSC-derived materials.

7.1.3. Materials

7.1.3.1. Isolation of MSCs from whole marrow and cell culture

1. Whole bone marrow aspirate: can be obtained commercially (Lonza) or from a consented human donor.
2. Phosphate Buffered Saline (PBS). (See Note 1)
3. Ficoll-Paque Density 1.077g/mL (GE Healthcare).
4. Table-top centrifuge capable of 1500g.
5. Hemocytometer and microscope, or other method capable of determining cell count.
6. Human MSC medium: α-Minimum Essential Medium Eagle (Sigma Aldrich) supplemented with 15% (v/v) Fetal Bovine Serum (HyClone), 2% (v/v) Penicillin Streptomycin Solution (GIBCO), 1 ug/ml Gentamicin (Sigma Aldrich), 1 ng/ml bFGF (R&D Systems). Store at 4°C.
7. 37°C incubator.

7.1.3.2. Collection, concentration, and storage of MSC-CM

1. Conditioning Medium: Dulbecco's Modified Essential Medium (Sigma Aldrich) supplemented with 0.5% (w/v) bovine serum albumin, and 2% (v/v) Penicillin Streptomycin Solution (GIBCO).
2. 1X Trypsin prepared from 10X 0.5% Trypsin-EDTA (GIBCO).
3. Hemocytometer.
4. For small volume of MSC-CM: 3kDa Centrifugal Filters (Amicon Ultra Ultracel-3K, Cat No. UFC800396). Centrifuge capable of spinning at 4000g.
5. For large volume of MSC-CM: Amicon pressure concentrator (no longer manufactured), pressurized nitrogen gas and 3 kDa regenerated cellulose ultrafiltration membranes (Millipore Ultracel).
6. +4°C freezer for storage.

7.1.3.3. Preparation of MSC lysate

1. Sonicator.
2. Benchtop Centrifuge capable of 1500g.
3. +4°C freezer for storage.

7.1.3.4. Isolation of PBMC's from whole blood

1. Fresh whole blood from consenting donor.
2. PBS.
3. Ficoll-Paque Density 1.077g/mL (GE Healthcare).
4. C-10 medium: 500 mL 1X RPMI Medium (GIBCO 1650), 50 mL inactivated Fetal Bovine Serum, 6 mL MEM Non-Essential Amino Acids Solution 10mM 100X (GIBCO Cat No. 1140-050), 6 mL 100X Sodium Pyruvate (GIBCO 11360), 6 mL glutamate, 6 mL Penicillin Streptomycin Solution (GIBCO 15140), 6 mL Sodium Bicarbonate, 3 mL Betamercaptoethanol. Sterilize with Corning Bottle Top Filter or other means of sterilization. Store at 4°C.
5. Centrifuge capable of spinning at 1500g.
6. 96-well plate.

7. 37°C incubator.

7.1.3.5. Preparation of samples and stimulation with LPS

1. 96-well plate seeded with 50 µL per well at $2x10^6$ PBMCs/mL.
2. MSC-CM and lysate, 50µL per well.
3. LPS (E. coli 0111:B4, Sigma-Aldrich L4391) at 30µg/mL, 50µL per well. Stock solution is diluted in C10 medium.
4. 37°C incubator.
5. -80°C freezer.

7.1.3.6. ELISA and Analysis of Results

1. BD OptEIA™ Human IL-10 ELISA Set and recommended buffers and solutions, or other Human IL-10 ELISA kit.
2. BD Falcon™ Microtest™ 96-Well ELISA Plate or other high-binding ELISA Plate.
3. Plate reader (spectrophotometer) capable of reading at indicated wavelengths in ELISA kit (450 nm with correction at 570 nm for BD OptEIA™ Human IL-10 ELISA set).
4. Microsoft Excel, or other program capable of processing data from spectrophotometer.

7.1.4. Methods

Overview: MSC-CM or lysate is prepared and added to PBMCs freshly isolated from whole blood and plated in a 96-well plate. As a mock cell control, we perform the assay with CM and/or lysates from fibroblasts. The plate is incubated overnight at 37°C for 16-18 hours. The PBMCs are then stimulated with LPS for 5 hours, at which time the plate is centrifuged and the supernatant stored for ELISA (**See Figure A2**).

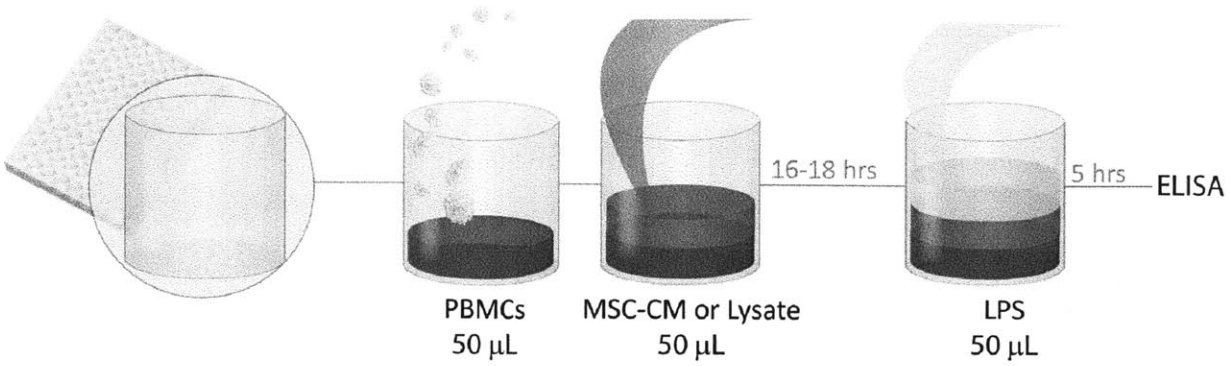

Figure A2 – Summary of In Vitro Inflammation Assay for the testing of MSC-derived factors.

7.1.4.1. Isolation of MSC's from whole bone marrow

Whole bone marrow centrifuged with Ficoll results in a pattern comparable to that of centrifuged whole blood; the corresponding "buffy coat" is enriched with MSCs. This layer is collected, counted, and plated on tissue culture polystyrene. The purity of MSCs isolated from whole bone marrow is relatively high due to their ability to differentially adhere to cell culture substrates compared to other hematopoietic marrow cells. Subsequent medium changes eliminate hematopoietic cells and other non-adherent cells. The identity of MSCs can be confirmed through phenotype and multipotency analysis using flow cytometry or differentiation media, respectively. (See **Note 3**)

1. Wash bone marrow with equivalent volume of PBS, thus diluting the bone marrow 1:2. Prepare 5 mL Ficoll for each 10 mL of diluted bone marrow. Add diluted whole bone marrow slowly; avoid disturbance of the boundary between Ficoll and marrow. (See **Note 1**)
2. Spin 30 minutes at 1500gwith no brake. Collect the resulting mononuclear cell layer and wash with 5 mL PBS.
3. Spin 10 minutes at 1500g, high brake. Resuspend pellet in 1 mL hMSC medium. Determine cell count and seed at a density of 1-10x10^3 cells/cm^2.
4. Let cells adhere and grow for 10 days. Perform first medium change at day 10; the following medium change is done at day 17. Subsequent medium changes should be

performed at a frequency of every 3 or 4 days. Cells are typically used between passages 1-6.

7.1.4.2. Collection, concentration, and storage of MSC-CM

By convention, 1X MSC-CM is defined as conditioned medium from 2 million cells concentrated to 1 mL of volume. MSCs are incubated for 24 hours in conditioning medium and concentrated to 1X. For small volumes, centrifugal filter tubes can be used. For larger volumes, the Amicon pressure concentrator and pressurized nitrogen gas offers a more efficient method.

1. Perform medium change on MSC cultures with two PBS washes. Add conditioning medium, incubate for 24 hours. (See Note 4)
2. Collect MSC-CM. Trypsinize cells; determine cell count and final volume of 1X MSC-CM.

For small volumes:

3. Add 4 mL PBS to centrifugal filter tube. Let centrifuge spin to 4000g for a few minutes for the PBS to run through the filter to remove residual glycerin in the ultrafiltration membrane. Discard the PBS in both compartments of the centrifugal filters.
4. Add 4 mL MSC-CM under sterile conditions and spin at 4000g for 5-15 minutes. Discard flow through and fill the centrifugal filter tube with new MSC-CM. Pipette the conditioned medium up and down to wash the filter and lessen congestion of the filter with proteins. Repeat process until the desired volume is reached.

For large volumes:

5. Sterilize the ultrafiltration membrane with 70% ethanol and let dry. Assemble pressure concentrator according to manufacturer instructions. Place pressure concentrator onto stir plate and prepare a waste bottle.
6. Run 20 mL of PBS through the concentrator.

7. Discard waste and add MSC-CM. Monitor waste level in the bottle to determine the volume of MSC-CM.
8. Let run until desired final volume is reached. Disconnect and depressurize concentrator.

7.1.4.3. Preparation of MSC lysate

While cell lysate can be obtained through lysate buffers and other chemical means, sonication, which causes physical disruption of the cell membrane, provides pure lysate without chemical contaminant and possible confounding factors.

1. Trypsize MSCs and pellet in tabletop centrifuge at 1000g.
2. Discard supernatant and gently layer 1 mL PBS per 2X106 cells on top of cell pellet. To visualize lysis, do not resuspend cells into solution.
3. Sonicate cell pellet at 3 A.U. for 5 seconds with 3 pulses and collect in ice.
4. Centrifuge lysate at 2000g for 2 minutes to precipitate membrane fragments. Retain the solution phase. The solution phase from this process is considered MSC-Ly.

7.1.4.4. Isolation of PBMCs from whole blood

1. Calculate the number of wells needed to conduct the assay to estimate the amount of blood needed, leaving extra wells for standards and controls. 100,000 PBMCs will be required per well. Collect fresh whole blood from a consented donor. It is best to plate the PBMCs and add the MSC-CM or lysate on the day of the separation to ensure maximum viability. (See Note 2) We have found 5 mL of blood to safely supply 10-15 million PBMCs, but this count varies from donor to donor.
2. Wash blood with equivalent volume of PBS, thus diluting the blood 1:2. Prepare 5 mL Ficoll for each 10 mL of diluted blood. Gently layer diluted blood on the Ficoll column. (See Note 1)
3. Spin 30 minutes at 1500g with no brake. Collect buffy coat and wash with 5 mL PBS.

4. Spin 10 minutes at 1500g. Resuspend pellet in 1 mL C-10 medium and determine cell count.
5. Dilute or concentrate to 2 million cells/ mL. Seed in 96-well plate at 50 µL per well (100,000 cells per well). Incubate at 37°C for storage if MSC-CM or lysate cannot be added immediately.

7.1.4.5. Preparation of samples and stimulation with LPS

1. Add 50 µL of MSC-CM or lysate to each well in the blood plate and incubate at 37°C for 16-18 hours.
2. Prepare LPS solution. Vortex well before dilution. Prepare 6 mL per plate of LPS diluted in C-10. Add 50 µL to each well, thus yielding a final concentration of 10 µg/mL in the wells, and incubate at 37°C or 5 hours.
3. Spin plates at 1500g for 10 minutes to settle PBMCs to the bottom of the wells. Collect supernatant in a new 96-well plate and store at -80°C. (See Note 5)

7.1.4.6. ELISA and Analysis of Results

1. Perform ELISA for human IL-10 according to manufacturer instructions. Use spectrophotometer to read plates at wavelengths indicated by ELISA kit and import raw data into program of choice.
2. Perform linear regression to generate standard curve and equation.
3. Convert raw absorbance data into concentration values using linear regression equation and generate bar graph. High IL-10 secretion by PBMCs indicates high potency of treatment.

7.1.5. Conclusions

MSCs are promising candidates for cell-based immunomodulatory therapy. They can be easily isolated from bone marrow aspirates, expanded 50 population doublings in 10 weeks with minimal loss in potency, and to date have not been found to cause adverse

immune responses in allogenic transplantation recipients [396]. Despite controversial theories regarding the primary therapeutic mechanism of action, the uses of MSC treatments have become diverse [392]. In our lab, we have demonstrated the effective use of MSC-CM and MSC-Ly in treating multiple organ dysfunction syndrome. To harness both the secreted and intracellular metabolism of MSCs, we have also created a MSC extracorporeal device for the treatment of organ failure [41, 42].

By quantifying the potency of MSC-CM or MSC-Ly, this method enables optimization of dosage, growth and storage conditions, as well as treatment procedures for clinical use of MSCs and MSC-based products. In future development of these products, the anti-inflammatory activity that these cells possess can be measured reliably and reproducibly with this assay, providing for better consistency and more rigorous release criteria. The assay also provides a valuable tool in elucidating the mechanism underlying MSC immunomodulation. The speed at which MSC transplantation conveys therapeutic activity (~ hours) is considerably faster than that of other cell transplants for regeneration purposes (~days-weeks), an inconsistency that can only be explained if other hypotheses for the mechanism of action other than engraftment and differentiation are considered. The time scale of these results could plausibly be explained by the occurrence of MSC lysis and the release of what would be paracrine factors during transplantation. This potency assay can account for and facilitate decomposing the "lysate effect".

7.1.6. Notes

1. It is recommended that the bone marrow or blood be diluted 1:1 with PBS, as the larger volume provides a greater margin of error during the collection of the buffy coat.
2. We found that blood stored for prolonged periods before use tends to be activated during storage and can thereby confound the results of the assay. Freshly obtained PBMCs are preferred.
3. Phenotype and Multipotency Analysis

While the identities of relevant cell surface markers of human MSCs remain controversial, Table 1 offers a certain immunophenotype of MSCs. It is possible to label for certain cell surface markers and conduct flow cytometry for isolation.

Table A1 – Immunophenotype of Human MSCs

Positive	Negative	Inducible
CD13, CD29, CD44, CD49a, b, c, e, f, CD51, CD54, CD58, CD71, CD73, CD90, CD102, CD105, CD106, CDw119, CD120a, CD120b, CD123, CD124, CD126, CD127, CD140a, CD166, P75, TGF-bIR, TGF-bIIR, HLA-A, B, C, SSEA-3, SSEA-4, D7, PD-L1	CD3, CD4, CD6, CD9, CD10, CD11a, CD14, CD15, CD18, CD21, CD25, CD31, CD34, CD36, CD38, CD45, CD49d, CD50, CD62E, L, S, CD80, CD86, CD95, CD117, CD133, SSEA-1, ABO	HLA-DR

Note: CD, cluster of differentiation; TGF, transforming growth factor; HLA, human leukocyte antigen; SSEA, stage-specific embryonic antigen; ABO refers to blood group antigens

MSCs have been shown to be capable of differentiating into bone, cartilage, adipose cells, and myoblasts in vivo [392]. Lee et al. developed differentiation media in which, under the right conditions, MSCs can be observed to differentiate into these tissues in vitro [397]. Table 2 includes specialized media used to encourage differentiation of MSCs. MSC differentiation kits are also commercially available from vendors.

Table A2 – Differentiation Media for MSCs

Differentiation Medium	Composition
Osteogenic	IMDM with 0.1 µM dexamethasone (Sigma-Aldrich, St Louis, MO), 0.2 mM ascorbic acid (AsA; Sigma-Aldrich), 10 mM -glycerol phosphate (Sigma-Aldrich).
Chondrogenic	high-glucose DMEM (Bio-fluid, Rockville, MD) with 0.1 M dexamethasone, 50 g/mL AsA, 100 g/mL sodium pyruvate(Sigma-Aldrich), 40 g/mL proline (Sigma-Aldrich), 10 ng/mL TGF- 1, and 50 mg/mL ITS premix (Becton Dickinson; 6.25 g/mL insulin, 6.25 g/mL transferrin, 6.25 ng/mL selenius acid, 1.25 mg/mL bovine serum albumin (BSA), and 5.35 mg/mL linoleic acid).
Adipogenic	IMDM with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1 M hydrocortisone (Sigma-Aldrich), 0.1 mM indomethacin(Sigma-Aldrich), and 10% rabbit serum (Sigma-Aldrich).

It is also possible to perform multipotency tests in vivo through subcutaneous transplantation of MSCs. While it is beyond the scope of this paper to discuss in vivo assays, the reader may find it worthwhile to refer to the work of Bianco and colleagues concerning the formation of ectopic bone marrow using subcutaneous transplants of MSCs [398].

4. This volume is typically 15 mL for Corning T-175 Flasks. For other containers a similar volume per cell ratio should be achieved, although small variations are negligible as the final definition of 1X and 10X medium are based on cell count and not initial volume.
5. Despite centrifugation, the supernatant may still be contaminated with PBMCs during the collection process. Freezing before the ELISA is recommended to lyse all remaining cells.